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Endocrine Control of Mammary-Gland Development and Function in the C3H/He Crgl Mouse[1,2]

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Summary

Normal mammary-gland development, including prepubertal and postpubertal changes and changes occurring during pregnancy and lactation, was described for the C3H/He Crgl female mouse. Hormonal control of the various phases of mammary development was studied in hypophysectomized-ovariectomized and hypophysectomized-ovariectomized-adrenalectomized mice. The effects of exogenous ovarian, adrenocortical, and adenohypophysial hormones, administered singly and in various combinations, were observed. Ductal development comparable to that seen in prepubertal and early postpubertal females was induced by a combination of β-estradiol (E) plus deoxycorticosterone acetate (DCA) plus cortisol acetate (F) plus bovine growth hormone (STH). Ductal and alveolar development similar to the adult virgin condition was induced by treatment with E plus progesterone (P) plus 100 μg. ovine mammotropic hormone (MH) or STH. Mammary development such as that found in early pregnancy was induced by treatment with E plus P plus DCA plus MH or STH. Mammary development comparable to that seen in the 2nd week of pregnancy was induced by treatment with E plus P plus DCA plus F plus STH or with E plus P plus DCA plus MH plus STH. In animals possessing full lobulo-alveolar development, subsequent treatment with F plus MH plus STH proved to be the most effective lactogenic combination, although some lactogenesis occurred after treatment with F and either protein hormone, alone. DCA was unable to replace the lactogenic activity of F in the above combinations. STH was as effective as MH in the induction of comparable stages of mammary-gland development in the C3H/He Crgl mouse.—**J. Nat. Cancer Inst. 21: 1039–1063, 1958.**

The mammary apparatus of the female laboratory mouse, *Mus musculus*, consists of 5 pairs of flat glands in the thoracic and abdomino-inguinal regions. The changes occurring in these glands during the life of the

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animal can be conveniently divided into the following phases: pre- and postpubertal development leading to the adult virgin condition; changes that take place during pseudopregnancy, pregnancy, lactation, and postlactational regression; and senile involution. The C3H/He Crgl strain of mice carries the mammary-tumor agent and has a mammary-tumor incidence of nearly 100 percent in nulliparous as well as in multiparous females. Two additional phases of mammary development must therefore be considered in mice of this strain. The first of these is the formation of hyperplastic alveolar nodules, which are usually found in the mammary glands of mature virgins and parous females. Considerable attention has been paid to the characterization and significance of these hyperplastic nodules and it is generally accepted that they are preneoplastic lesions (*1–8*). The other recognizable developmental phase is represented by the formation of mammary carcinomas.

Various aspects of mammary-gland development in intact mice of several strains have been studied by Turner and Gomez (*9*), Cole (*10*), Wieser (*11*), and Fekete (*12*). However, with the exception of the pioneer studies of Selye *et al.* (*13*) and Gardner (*14–16*), and the recent work by Ferguson (*17*), hypophysectomized mice have not been employed in the analysis of mammary-gland development. On the other hand, the hormonal control of various phases of mammary morphogenesis in the rat has been worked out by several investigators (*18*, *19*) who have utilized extensively the hypophysectomized animal.

The present report summarizes experiments on the endocrine control of mammary-gland development and function in the female C3H/He Crgl mouse, which are reported in detail in a monograph published as a "University of California Publication in Zoology" (*20*). In addition, the results of some subsequent experiments on the action of ovarian, adrenocortical, and adenohypophysial hormones are included at this time. The over-all aim of the investigation has been to arrive at an understanding of the hormonal control of normal mammary-gland development in the mouse in order to elucidate eventually the role of hormones in preneoplastic and neoplastic changes. Notwithstanding the existence of the earlier studies referred to, a survey of normal postnatal mammary-gland development in our female C3H/He Crgl mice was conducted in order to control for strain-specific features and to establish a series of normal standards with which the experimental material could be compared. In addition, the complete exposition of normal mammary development (*20*) includes data on body- and organ-weight changes that are not presented herein. The endocrine control of mammary-gland development was analyzed experimentally by extirpation of the ovaries and the adrenal and pituitary glands, singly and in various combinations, and by subsequent replacement therapy.

Methods and Materials

This report is based on the study of mammary glands from 652 female C3H/He Crgl mice that survived the full experimental periods. How-

ever, tables 2 through 5, which summarize the survival rates and the mammary-gland responses of various experimental groups, include only the experimental series that have been considered in this report. For the study of normal mammary development, 155 intact animals were used, which ranged in age from 1 day to 12 months; each group consisted of 4 to 20 mice. The experimental animals were all virgin females 12 weeks of age at the start of experimental procedures, with 4 to 14 mice per group.

In all experiments involving induction of mammary development with exogenous hormones, the animals were hypophysectomized-ovariectomized or hypophysectomized-ovariectomized-adrenalectomized. At 12 weeks of age, ovariectomy and combined ovariectomy-adrenalectomy were performed via the dorsal approach and preceded hypophysectomy by 3 to 5 days. The details of the operative procedures and the postoperative care of the animals are discussed elsewhere (*20*, *21*).

The hormones used in these experiments (table 1) include: 2 ovarian steroids (β-estradiol, E; progesterone, P), 3 adrenocortical steroids (corticosterone acetate, B; cortisol acetate, F; deoxycorticosterone acetate, DCA), and 2 adenohypophysial hormones (bovine growth hormone, STH; ovine mammotropic hormone, MH). Biologic assay of MH and STH demonstrated the presence of less than 1 percent MH in STH and *vice versa*. The hormones were administered in daily subcutaneous injections of suspension (steroids) or solution (proteins), and

TABLE 1.—*Source, preparation, and dosages of hormones*

Hormone	Method of use	Dose	Source
β-Estradiol (E)	(1) Subcutaneously implanted pellet (2) Aqueous suspension of microcrystals	2–3 mg. 0.1–2.5 μg./day	Schering Corporation (Progynon)
Progesterone (P)	Crystalline, in aqueous suspension	1 mg./day	Schering Research Division, Lots #5RMA86, #6RMA62, #7RMA30
Corticosterone acetate (B)	Crystalline, in aqueous suspension	125 μg./day	Merck Sharp & Dohme, Lot #57R1263
Cortisol acetate (F)	Saline suspension	62.5–200 μg./day	Merck Sharp & Dohme (Hydrocortone acetate)
Deoxycorticosterone acetate (DCA)	Crystalline, in aqueous suspension	200–500 μg./day	Schering Research Division, Lot #DC-3-LL-LV
Mammotropin (MH)	Aqueous solution at various *p*H's (7.6–10.5)	0.1 mg.–1 mg./day	Prepared from ovine pituitaries according to the method of Cole and Li (*22*), Lots #L2641A, #L2738E
Growth hormone (STH)	Aqueous solution at various *p*H's (8.0–10.5)	0.1 mg.–1 mg./day	Prepared from bovine pituitaries according to the method of Li (*23*), Lot #L2732D-A

hormonal treatment was always begun on the day following the completion of surgical procedures. Whenever more than one steroid hormone was administered, the hormones were combined in a single injection in a volume of 0.2 ml. Similarly, mixtures of pituitary hormones were made when these were administered simultaneously.

In all experiments, the animals were killed 24 hours after the final injection. At necropsy, the body weight and weights of the adrenals, thymus, and spleen were recorded. All animals were examined carefully under a dissecting microscope to insure the completeness of the operations. Completeness of hypophysectomy in representative animals was further checked by serial sections through the hypophysial capsule and adjacent tissues, including the pituitary stalk and median eminence. Only animals that showed no evidence of residual pituitary tissue were used in the final analysis of the results.

The integument with all 5 pairs of mammary glands attached was fixed overnight in 10 percent formalin. The formalin-fixed mammary glands were dissected off the skin, stained with iron hematoxylin (modified from Craig and Wilson, *24*), and stored and photographed as whole-mounts in methyl salicylate. The degree of mammary development was assessed from these whole-mount preparations, which were examined under a dissecting microscope. Segments of representative glands were removed from the bulk-stained whole-mount preparations, sectioned, and stained further with a modified Masson's trichrome stain for histologic study. Details of these techniques and the criteria adopted for the study of mammary-gland growth and development are described elsewhere (*20*).

Some variation in mammary-gland morphology was noted between different glands in the same animal and between different animals in the same group. The descriptions and the photomicrographs are representative of the average mammary development observed. It should be emphasized that only slight variation was noted in individual response to any stated experimental procedure. All photomicrographs of whole-mount preparations were made from one of the third pairs of thoracic glands.

Results

Normal Mammary-Gland Development

The observations reported herein and summarized in table 2 represent a brief synopsis of the principal features of normal mammary-gland development in the C3H/He Crgl mouse. Further details are presented elsewhere (*20*).

Prepubertal ductal development.—Prepubertal ductal proliferation begins between the 3rd and 4th week of life. At this time, club-shaped terminal buds are formed at the free ends of the ducts (fig. 1). Elongation of the duct system, with the formation of additional club ends, results in further growth of the mammary gland. In females from 6 to 7 weeks of age, the mammary glands do not yet occupy the entire fat pad.

Postpubertal ductal development.—Postpubertal ductal growth begins

between the 8th and 9th week of age, and the fat pads are soon filled with the mammary-duct system. At this stage, the glands consist of wide ducts and terminal buds of different sizes, including club ends.

Ductal and alveolar development.—The mammary glands in 75 percent of 12-week-old females (fig. 2) appear similar to those of 9- to 10-week-old females. In 25 percent of these animals, however, the glands consist of thin branching ducts, some alveolar buds, and a few isolated alveoli (fig. 3).

By the 16th week of age, the mammary glands show more extensive duct branching, and most glands contain numbers of isolated alveoli (fig. 4). This was the maximum development observed in virgin females that were not pseudopregnant.

Examination of vaginal smears along with the mammary glands from 8- to 12-week-old females indicated some changes in mammary-gland morphology at various stages of the estrous cycle. However, no correlation could be made between the stages of the estrous cycle and the mammary-gland changes in 16-week-old virgin females.

Beginning lobulo-alveolar development.—During the first week of pregnancy (fig. 5), the mammary glands showed further ductal branching and an increase in the number of alveoli. Some of the alveoli were organized into small lobules at this stage.

Full lobulo-alveolar development.—During the 2nd week of pregnancy, the lobulo-alveolar development progresses steadily, with a further increase in the number and size of the alveoli (fig. 6). By the end of this period the formation of new alveoli ceases.

Lactogenesis.—Between the 17th and 19th day of pregnancy, localized areas of lactogenesis appear in the mammary glands. Histologically, the alveoli in these areas are composed of a layer of highly vacuolated cuboidal cells with secretory material filling the alveolar lumina.

Lactational changes.—For approximately 12 days after parturition, the amount of milk in the mammary glands increases, with a concomitant increase in the thickness of the glands. Between 3 and 12 days post partum, there is a decrease in the degree of cell vacuolization and an increase in the amount of secretory material in the alveolar lumina (figs. 7, 8, and 9). No differences were observed between the glands of 12- and of 21-day postpartum females.

Postlactational involution.—The mammary glands of 2-day postweaning females are thick owing to congestion of milk in the alveoli. Histologically, the alveolar lumina are dilated and filled with secretion. Some collapsed alveoli were also visible in parts of these glands.

By the 5th day after weaning, the mammary glands are devoid of milk and the alveoli are reduced to masses of cells without lumina.

Preneoplastic and neoplastic transformations.—In 12-month-old multiparous females, the mammary glands contain large numbers of hyperplastic alveolar nodules and 1 or more mammary tumors of various sizes. Results of experiments on the hormonal induction of precancerous nodules and mammary tumors have been reported elsewhere (*25*).

TABLE 2.—*Mammary-gland development in intact female C3H/He Crgl mice*

Group	Number of mice	Age or state at autopsy	State of mammary-gland development
1 2 3	6 6 6	4 weeks 6 weeks 7 weeks	Glands consist of medium and wide ducts with small and large terminal buds and do not occupy the entire fat pad
4 5	5 5	8 weeks 9 weeks	Glands occupy the entire fat pad; wide ducts with large numbers of terminal buds
6	20	12 weeks	Majority of mice possess mammary glands with wide ducts and terminal buds; 25% of the mice had thin ducts with alveolar buds and few alveoli
7	24	16 weeks	Highly developed duct system with isolated alveoli
8	4	1st week of pregnancy	Increase in gland size and duct branching with some lobulo-alveolar development
9	4	2nd week of pregnancy	Further increase in lobulo-alveolar development
10	4	18th day of pregnancy	Increase in size of lobules; localized lactogenesis
11	4	3 days post partum	Glands are considerably thicker and completely filled with milk
12	4	12 days post partum	Further increase in the thickness of milk-filled glands
13	4	21 days post partum	Same as in group 12
14	4	2 days post weaning	Same as in groups 12 and 13
15	4	5 days post weaning	Complete absence of milk; alveoli regressed
16	5	Multiparous tumor-bearing females (12 months old)	Highly branched duct system with occasional alveoli; small tumors and hyperplastic nodules

Effect of Extirpation of Endocrine Glands on Mammary-Gland Morphology

Twelve-week-old virgin females were used in these experiments. One month after surgical procedure the animals were subjected to routine necropsy. The following operations were performed on different groups of animals: 1) ovariectomy, 2) adrenalectomy, 3) hypophysectomy, 4) ovariectomy plus adrenalectomy, 5) hypophysectomy plus ovariectomy, 6) hypophysectomy plus adrenalectomy, and 7) hypophysectomy plus ovariectomy plus adrenalectomy.

Examination of the mammary glands of these animals indicated that alveoli and terminal buds were not maintained in the absence of either the ovaries or the pituitary (fig. 10). Adrenalectomy had little, if any, effect on mammary-gland morphology, but the alveolar development in the adrenalectomized animals was, in general, slightly less than that in normal 16-week-old females. Regression of ductal branching and of the

alveoli occurred only in animals from which both ovaries and adrenals were removed (fig. 11).

Experimental Induction of Mammogenesis and Lactogenesis in Hypophysectomized-Ovariectomized and Hypophysectomized-Ovariectomized-Adrenalectomized C3H/He Crgl Female Mice (see *tables 3, 4, and 5*)

Twelve-week-old virgin females were hypophysectomized and ovariectomized, or hypophysectomized, ovariectomized, and adrenalectomized at the start of these experiments. The following hormonal combinations were used in various groups of animals: 1) ovarian steroids alone, 2) adrenocortical steroids alone, 3) ovarian plus adrenocortical steroids, 4) adenohypophysial hormones alone, 5) ovarian plus adenohypophysial hormones, and 6) ovarian plus adrenocortical plus adenohypophysial hormones combined.

Ductal development.—β-Estradiol (E), when given in a daily dose of 0.1 μg. or 1.0 μg. for 30 days, was found to induce slight ductal proliferation in hypophysectomized-ovariectomized females. When the estradiol was administered as a 2 to 3 mg. pellet and implanted subcutaneously, the mammary glands were found to be quite small and contained fine ducts. The cells composing these ducts had pyknotic nuclei.

Growth hormone (STH), at a dose of 1 mg. per day, resulted in greater mammary-duct branching than was found in intact 12-week-old females.

Cortisol acetate (F) maintained the duct branching in hypophysectomized-ovariectomized females when 125 μg. per day was administered for 30 days. If the dose of F was increased to 200 μg. per day, an atypical stimulation of duct branching occurred. The ducts became very fine and highly branched, and histologic examination revealed that the lumina of these ducts were reduced or entirely absent, and the nuclei of many epithelial cells were pyknotic. F-treated animals had larger mammary fat pads compared to intact females of the same age. With the exception of these groups in which the fat pads were obviously large, no special attention was paid to the size of the fat pads in other experimental groups.

Mammotropic hormone (MH), at a dose of 1 mg. per day, was ineffective in preventing mammary regression in hypophysectomized-ovariectomized females. Similarly, deoxycorticosterone acetate (DCA), 500 μg. per day, had no appreciable effect on these mammary glands.

Several steroid combinations stimulated duct branching in hypophysectomized-ovariectomized and in hypophysectomized-ovariectomized-adrenalectomized animals treated for 30 days. These included (figures in parentheses indicate daily doses): E (2.5 μg.) plus DCA (500 μg.) plus corticosterone acetate (B, 125 μg.); E (2.5 μg.) plus progesterone (P, 1 mg.) plus F (125 μg.); and E (2.5 μg.) plus P (1 mg.) plus DCA (500 μg.) plus B or F (125 μg.). The last two combinations resulted in maximal duct branching in hypophysectomized-ovariectomized-adrenalectomized females.

Some combinations in which pituitary hormones were also used resulted in ductal proliferation. E (2.5 μg.) plus STH (1 mg.) stimulated some duct branching in hypophysectomized-ovariectomized females. E plus MH was ineffective, however. Duct stimulation was also obtained in hypophysectomized-ovariectomized-adrenalectomized females treated for 20 days with any of the following combinations: E (1 μg.) plus DCA (200 μg.) plus MH or STH (0.5 mg.); E (1 μg.) plus DCA (200 μg.) plus F (62.5 μg.) plus MH or STH (0.5 mg.). It was noted that combinations that include F resulted in more extensive ductal proliferation than those that omit F (figs. 12 and 13).

Simultaneous ductal and alveolar development.—The mammary glands of hypophysectomized-ovariectomized animals treated with E (1 or 2.5 μg.) plus P (1 mg.) plus DCA (500 μg.) for 30 days showed some stimulation of ductal branching accompanied by the presence of a few isolated alveoli (fig. 14). Mammary glands similar to those of adult virgins (16 weeks of age and older), with some isolated alveoli and occasional lobules, were induced in hypophysectomized-ovariectomized females with E (1 μg.) plus P (1 mg.) plus STH or MH (0.1 mg.) (figs. 15 and 16).

Beginning lobulo-alveolar development.—Treatment of hypophysectomized-ovariectomized females with E (2.5 μg.) plus P (1 mg.) plus DCA (500 μg.) plus MH (1 mg.), or hypophysectomized-ovariectomized-adrenalectomized females with E (2.5 μg.) plus P (1 mg.) plus DCA (500 μg.) plus STH (1 mg.) for 30 days induced mammary development similar to that seen in the first week of pregnancy (fig. 17). Both these combinations acted chiefly to induce duct branching and the formation of alveoli. Replacement of the DCA with F resulted in less duct branching and fewer alveoli, but the alveoli were generally larger.

Full lobulo-alveolar development.—Mammary development similar to that seen during mid-pregnancy could be induced by treating hypophysectomized-ovariectomized and hypophysectomized-ovariectomized-adrenalectomized animals with E (1 or 2.5 μg.) plus P (1 mg.) plus STH and/or MH (1 mg.) for 30 days (figs. 18 and 19). Similar results were also obtained by treating the hypophysectomized-ovariectomized-adrenalectomized animals with E (1 μg.) plus P (1 mg.) plus DCA (500 μg.) plus F (125 μg.) plus STH (1 mg.) for 30 days (fig. 21). Mammary glands of hypophysectomized-ovariectomized-adrenalectomized females treated with E (1 μg.) plus P (1 mg.) plus DCA (500 μg.) plus MH (1 mg.) plus STH (1 mg.) showed even greater increase in the size and number of lobules, approaching the condition seen at the end of the 2nd week of pregnancy (fig. 20).

Localized and complete lactogenesis.—Localized lactation could be induced in some of the hypophysectomized-ovariectomized-adrenalectomized females with E (2.5 μg.) plus P (1 mg.) plus F (125 μg.) plus STH (1 mg.). If DCA (500 μg.) was added to this combination, then more alveoli were found, but the individual alveoli were smaller and contained less secretion.

Lactogenesis occurred in parts of the mammary glands of hypophysectomized-ovariectomized-adrenalectomized animals treated with E (2.5

μg.) plus P (1 mg.) plus F (62.5 μg.) plus MH (1 mg.) plus STH (1 mg.) for 30 days. The secretory areas of these glands were indistinguishable from glands of intact 12-day postpartum females.

Attempts were then made to induce lactation in hypophysectomized-ovariectomized and hypophysectomized-ovariectomized-adrenalectomized females by treating them first with hormones designed to stimulate lobulo-alveolar development, and later with combinations that would induce lactation in well-developed lobules of alveoli. The following treatments resulted in lactogenesis in these animals:

1) E (1 μg.) plus P (1 mg.) plus MH (1 mg.) for 15 days, followed by MH (1 mg.) plus F (125 μg.) for 5 days (fig. 22).

2) E (1 μg.) plus P (1 mg.) plus STH (1 mg.) for 15 days, followed by STH (1 mg.) plus F (125 μg.) for 5 days (fig. 23).

3) E (1 μg.) plus P (1 mg.) plus MH (1 mg.) plus STH (1 mg.) for 15 days, followed by MH (1 mg.) plus F (125 μg.) for 5 days.

4) E (1 μg.) plus P (1 mg.) plus MH (1 mg.) for 15 days, followed by STH (1 mg.) plus F (125 μg.) for 5 days.

5) E (1 μg.) plus P (1 mg.) plus DCA (500 μg.) plus F (62.5 μg.) plus MH (1 mg.) plus STH (1 mg.) for 15 days, followed by MH (1 mg.) plus STH (1 mg.) plus DCA (500 μg.) plus F (125 μg.) for 5 days.

TABLE 3.—*Effect of removal of pituitary, ovaries, and adrenals, singly and in combinations, on the mammary glands of 12-week-old C3H/He Crgl mice**

Group	Operation performed†	Initial No. of mice	Number of surviving mice	State of mammary-gland development
17	$\overline{O}$	10	10	Glands consist of bare ducts only; fat pads larger than those of intact animals of the same age
18	$\overline{A}$	10	10	No effect; mammary glands as in group 7
19	$\overline{H}$	12	11	Ducts thin; complete regression of alveoli and terminal buds
20	$\overline{O}$-$\overline{A}$	10	6	Regression of ductal branching, terminal buds, and alveoli
21	$\overline{H}$-$\overline{O}$	24	21	Same as in group 17 except for thinner ducts
22	$\overline{H}$-$\overline{A}$	10	7	Same as in group 19
23	$\overline{H}$-$\overline{O}$-$\overline{A}$	24	12	Same as in group 20; ducts usually very thin

*Examined 1 month postoperatively.
†$\overline{O}$=ovariectomy; $\overline{A}$=adrenalectomy; $\overline{H}$=hypophysectomy.

TABLE 4.—*Effect of ovarian, adrenocortical, and adenohypophysial hormones, singly and in combinations, on the mammary glands of female C3H/He Crgl mice, hypophysectomized + ovariectomized ± adrenalectomized at the age of 12 weeks*

Group	Operations performed*	Initial No. of mice	Number of surviving mice	Daily hormonal treatment							Duration (*days*)	State of mammary-gland development
				E (*μg.*)	P (*mg.*)	DCA (*μg.*)	F (*μg.*)	B (*μg.*)	STH (*mg.*)	MH (*mg.*)		
24	$\overline{\overline{H}} + \bar{O}$	10	7	0. 1	0	0	0	0	0	0	30	Duct branching was better than in intact 12-week-old females
25	$\overline{\overline{H}} + \bar{O}$	10	8	1. 0	0	0	0	0	0	0	30	
26†	$\overline{\overline{H}} + \bar{O}$	50	7	2000–3000	0	0	0	0	0	0	30	Mammary glands as a whole regressed
27	$\overline{\overline{H}} + \bar{O}$	5	5	0	0	0	0	0	1	0	30	Duct branching better than in groups 24 and 25
28	$\overline{\overline{H}} + \bar{O}$	10	10	0	0	0	125	0	0	0	30	Duct branching maintained
29	$\overline{\overline{H}} + \bar{O}$	15	7	0	0	0	200	0	0	0	30	Atypical duct proliferation
30	$\overline{\overline{H}} + \bar{O} + \bar{A}$	10	8	0	0	500	0	0	0	0	30	Mammary glands regressed
31	$\overline{\overline{H}} + \bar{O}$	19	17	0	0	0	0	0	0	1	30	
32	$\overline{\overline{H}} + \bar{O}$	8	8	2. 5	0	500	0	125	0	0	30	Duct branching was better than in groups 24 and 25
33	$\overline{\overline{H}} + \bar{O} + \bar{A}$	5	5	2. 5	1	0	125	0	0	0	30	
34	$\overline{\overline{H}} + \bar{O} + \bar{A}$	9	8	2. 5	1	500	0	125	0	0	30	
35	$\overline{\overline{H}} + \bar{O} + \bar{A}$	9	9	2. 5	1	500	125	0	0	0	30	
36	$\overline{\overline{H}} + \bar{O}$	9	5	2. 5	0	0	0	0	0	1	30	No stimulation of duct branching
37	$\overline{\overline{H}} + \bar{O}$	9	5	2. 5	0	0	0	0	1	0	30	Duct branching stimulated
38	$\overline{\overline{H}} + \bar{O} + \bar{A}$	5	5	1. 0	0	200	0	0	0. 5	0	20	

39	$\overline{H} + \bar{O} + \bar{A}$	8	7	1.0	0	200	0	0	0	0.5	20	
40	$H + \bar{O} + \bar{A}$	5	5	1.0	0	200	62.5	0	0.5	0	20	
41	$\overline{H} + \bar{O} + \bar{A}$	7	7	1.0	0	200	62.5	0	0	0.5	20	
42	$\overline{H} + \bar{O}$	8	8	2.5	1	500	0	0	0	0	30	Few alveoli present
43	$\overline{H} + \bar{O}$	8	8	2.5	1	0	0	0	0.1	0	30	Mammary-gland development similar to 16-week-old virgin females
44	$H + \bar{O}$	7	6	2.5	1	0	0	0	0	0.1	30	
45	$\overline{H} + \bar{O}$	9	7	2.5	1	500	0	0	0	1	30	Ductal branching and alveolar development highly stimulated
46	$H + \bar{O} + \bar{A}$	10	10	2.5	1	500	0	0	1	0	30	
47	$\overline{H} + \bar{O} \pm \bar{A}$	25	22	2.5	1	0	0	0	1	0	30	Lobulo-alveolar development similar to 2-week pregnant females
48	$H + \bar{O} \pm \bar{A}$	25	19	2.5	1	0	0	0	0	1	30	
49	$\overline{H} + \bar{O}$	8	8	2.5	1	0	0	0	1	1	30	
50	$\overline{H} + \bar{O} + \bar{A}$	9	9	2.5	1	500	125	0	1	0	30	
51	$\overline{H} + \bar{O} + \bar{A}$	6	5	1.0	1	500	0	0	1	1	30	
52	$H + \bar{O} + \bar{A}$	8	8	2.5	1	0	125	0	1	0	30	Lactogenesis observed in some parts of the glands
53	$H + \bar{O} + \bar{A}$	10	10	2.5	1	0	125	0	1	1	30	

*Abbreviations as in table 3.

†2000 to 3000 μg. of β-estradiol pellets were implanted subcutaneously.

TABLE 5.—*Induction of lactation in the mammary glands of hypophysectomized + ovariectomized ± adrenalectomized 12-week-old C3H/He Crgl mice*†*

Group	Initial No. of mice	Number of surviving mice	Initial treatment ‡ (*12 to 15 days*)	Final treatment (*5 days*)	State of mammary gland
54	10	9	E + P + MH	MH + F	Lactation as in 3-day postpartum females
55	8	6	E + P + STH	STH + F	
56	8	4	E + P + MH + STH	MH + F	
57	8	6	E + P + MH	STH + F	
58	8	8	E + P + DCA + F + MH + STH	MH + STH + DCA + F	
59	7	7	E + P + F (62.5 μg.) + MH + STH	MH + STH + F	Lactation as in 12-day postpartum females
60	8	5	E + P + MH	MH + DCA	Large numbers of lobules of alveoli devoid of milk
61	8	5	E + P + STH	STH + DCA	

*All groups were hypophysectomized-ovariectomized-adrenalectomized except group 57 which was hypophysectomized-ovariectomized.

†Unless otherwise mentioned the following hormone doses were used: E, 1 μg. per day; P, 1 mg. per day; DCA, 500 μg. per day; F, 125 μg. per day; STH, 1 mg. per day; MH, 1 mg. per day.

‡In groups 59 to 61, the initial period of treatment was 12 days. In all other groups initial treatment was carried out for 15 days.

6) E (1 μg.) plus P (1 mg.) plus F (62.5 μg.) plus MH (1mg.) plus STH (1 mg.) for 12 days, followed by MH (1 mg.) plus STH (1 mg.) plus F (125 μg.) for 5 days (fig. 24).

The first 5 combinations induced mammary development comparable to the lactating glands observed during the 1st week after parturition. The 6th combination, however, resulted in development similar to 12-day postpartum females.

If F was replaced by DCA in the hormonal combinations used during the last 5 days of treatment, no lactation was induced.

Discussion

Three periods of rapid ductal development occur in the mammary glands of intact females. These occur before puberty, immediately after puberty, and during the 1st week of pregnancy. Prepubertal ductal growth and that seen just after puberty occur without simultaneous proliferation of alveoli. Several hormonal combinations were found to induce this type of ductal development in hypophysectomized-ovariectomized and hypophysectomized-ovariectomized-adrenalectomized C3H/He Crgl females. These included: E plus DCA plus B; E plus P plus F; E plus P plus DCA plus B or F; E plus STH; E plus DCA plus MH or STH; and E plus DCA plus F plus MH or STH. In young mice in which the mammary glands are undergoing this ductal development, the adrenal and body weights are increasing rapidly (*20*). At 4 weeks of age, when prepubertal development begins, the vagina opens as well, indicating the presence of ovarian estrogen at this stage. Yokoyama and Syoda, cited by Richardson (*26*), considered the prepubertal development in the Kasukabe strain of mice to be a result of steroid secretion by the ovaries of these young animals. At any rate, it seems reasonable to conclude that both STH and estrogen, as well as some adrenocortical steroids, are secreted normally during prepubertal development. There is little evidence for the presence of either MH or P, however. In fact, if both P and STH were secreted at this time, the mammary glands should contain alveoli, inasmuch as alveolar development was always induced in animals treated with combinations that included E plus P plus STH. It appears, therefore, that early ductal proliferation is a result of E and STH secretion, with adrenocortical hormones probably playing a synergistic role. This is in agreement with the report of Lyons *et al.* (*18*) that E plus DCA plus STH are required for ductal development in triply operated Long-Evans rats.

Mammary glands containing ducts and alveoli, such as those found in adult virgin females, were obtained in hypophysectomized-ovariectomized and hypophysectomized-ovariectomized-adrenalectomized females by treatment with E plus P plus DCA, E plus P plus 100 μg. MH, or E plus P plus 100 μg. STH. The last two combinations were the most effective in the induction of simultaneous alveolar and ductal development. Progesterone, along with other hormones, is generally considered to be indispensable for the formation of alveoli in the mammary glands of rats

(*27*) and mice (*28*). Since no combination that excluded P was able to induce alveolar development in C3H/He Crgl mice, it must be concluded that adult virgin females are secreting at least small amounts of luteotropic MH and P. Probably these are present in minimal quantities, however, since these animals have a very short luteal phase in their estrous cycle. Mammary development characteristic of mature virgins is seen at 16 weeks of age. At this age the C3H female mouse is continuing to grow (*20*), which indicates that STH must still be present. From these observations, it can be assumed that E, STH, and small amounts of P and MH contribute to the mammary development seen in adult virgin females. It should also be mentioned that the addition of F to the combinations E plus P plus MH, or E plus P plus STH, inhibited the formation of alveoli. DCA, on the other hand, enhanced both ductal and alveolar development. It is likely, therefore, that DCA-like substances from the adrenal cortex are more important than F-like substances in the mammary development of adult virgin females and early pregnant females. It should be mentioned, however, that neither F nor DCA is considered to be secreted in significant quantities by the mouse adrenal cortex, and are only used here as effective examples of so-called "glucocorticoids" and "mineralocorticoids," respectively. The adrenocortical steroids in the normal mouse are probably aldosterone and corticosterone (*29*).

Lobulo-alveolar development like that found early in the 2nd week of pregnancy could be induced in hypophysectomized-ovariectomized-adrenalectomized females treated with E plus P plus 1 mg. MH or STH. A similar effect of E plus P plus MH was described by Lyons *et al.* (*30, 31*) in the Long-Evans rat, but E plus P plus STH were unable to induce this type of development in the rat.

Although E plus P plus F plus STH were relatively ineffective in the stimulation of alveolar growth, the addition of DCA to this combination resulted in mammary development similar to that of females at midpregnancy. E plus P plus DCA plus MH plus STH stimulated better lobulo-alveolar development. Combinations that included F always induced localized milk formation, which does not normally occur to any great extent during the first 2 weeks of pregnancy. Hence, F-like compounds, viz., corticosterone, probably do not contribute greatly to mammary-gland development during this period.

Partial lactogenesis such as that found in late pregnancy could be induced by hormonal combinations that included F. The difference between the actions of DCA and F was demonstrated further by the fact that treatment with E plus P plus F plus STH resulted in more lactogenesis than the same combination with DCA added. This suggests that DCA may antagonize the lactogenic effect of F.

The minimal hormonal combination needed for the initiation of milk secretion in the fully developed mammary glands of Long-Evans rats is considered to be MH plus F (*18, 32*). Our experiments, however, have shown that STH can replace MH in the induction of lactogenesis in

hypophysectomized-ovariectomized-adrenalectomized C3H/He Crgl females (*see also* Nandi, *33*). Furthermore, a combination that included both adenohypophysial hormones, E plus P plus F plus STH plus MH, was far more effective than either hormone alone when administered with the same steroid combination.

Complete lactogenesis has been obtained by Lyons *et al.* (*18*) in the hypophysectomized-ovariectomized-adrenalectomized Long-Evans rat. These animals were first treated with E plus DCA plus STH to stimulate ductal development, then with E plus P plus DCA plus Prednisolone acetate plus STH plus MH to obtain complete lobulo-alveolar development, and finally with DCA plus Prednisolone acetate plus MH, which induced complete lactation. In our experiments with the C3H/He Crgl mouse, however, DCA was again found to antagonize the action of F in the induction of full lactation. Combinations that included F were more effective than those containing F plus DCA, whereas combinations which included only DCA were completely ineffective in stimulating lactogenesis. These results agree with those of Gaunt (*34*) and Gaunt, Eversole, and Kendall (*35*), who indicated the importance of glucocorticoids in the initiation of lactogenesis in rats.

Female mice show a true gain in body weight (corrected for the hypertrophic uteri and their contents) during the last week of pregnancy and during lactation, and corpora lutea of pregnancy are found in the ovary at this time (*20*). Also, a sudden drop in the weight of the thymus during lactogenesis and galactopoiesis may be related in part to an increased production of glucocorticoids. In the intact female, therefore, MH, STH, and F-like substances are apparently present during the periods when secretory activity occurs in the mammary gland. All of these factors are probably important in the stimulation and maintenance of milk secretion.

The results presented herein point to several differences between the Long-Evans rat, studied by Lyons and his collaborators (*18*, *30*, *31*), and the C3H/He Crgl mouse with respect to the hormonal control of mammary-gland development and function. These differences can be summarized as follows:

1) In the C3H/He Crgl mouse, combinations of ovarian and adrenocortical steroids induce some ductal and alveolar development. Combinations of steroids alone appear to be ineffective in the rat.

2) Bovine STH is as effective as ovine MH in the stimulation of all phases of mammary-gland development in the mouse. In the rat, however, STH in the absence of MH is ineffective in the induction of lobulo-alveolar development or in lactogenesis.

3) Progesterone can act both as a ductal and as an alveolar mammogen in the mouse, particularly in the induction of ductal development comparable to that found in early pregnancy.

4) DCA can act as a lactogen in certain strains of rats. DCA has no lactogenic potency in the C3H/He Crgl mouse, and may even inhibit the action of F in stimulating mammary secretion.

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The figures in the accompanying plates show whole-mounts of representative areas in one of the third pairs of thoracic mammary glands from C3H/He Crgl female mice, stained with hematoxylin, and histologic sections stained with Masson's trichrome stain. With the exception of figure 1 (10.5×), the whole-mounts were photographed at a magnification of 7×. Sections were photographed at a magnification of 400×. Animals treated with various hormonal combinations were doubly or triply operated at 3 months of age.

The following abbreviations were used: doubly operated = hypophysectomized + ovariectomized; triply operated = hypophysectomized + ovariectomized + adrenalectomized; E = β-estradiol; P = progesterone; DCA = deoxycorticosterone acetate; F = cortisol acetate; STH = growth hormone; MH = mammotropin.

All the photomicrographs are by Mr. Virgil Finster of our laboratory.

PLATE 107

FIGURE 1.—Four-week-old intact mouse. *Note* the presence of club-shaped terminal buds.

FIGURE 2.—Twelve-week-old intact virgin female, showing the typical mammary-gland development.

FIGURE 3.—Twelve-week-old intact virgin female, representing a maximally developed mammary gland. *Note* the increased duct branching compared to figure 2, and the presence of alveolar buds.

FIGURE 4.—Maximally developed gland of a 16-week-old virgin mouse. This mammary gland contains a large number of isolated alveoli.

FIGURE 5.—Mammary gland during the 1st week of a first pregnancy, showing increased duct branching and alveolar development as well as a few beginning lobules of alveoli.

FIGURE 6.—Mammary gland of a female during the 2nd week of pregnancy. *Note* the large number of lobules of alveoli.

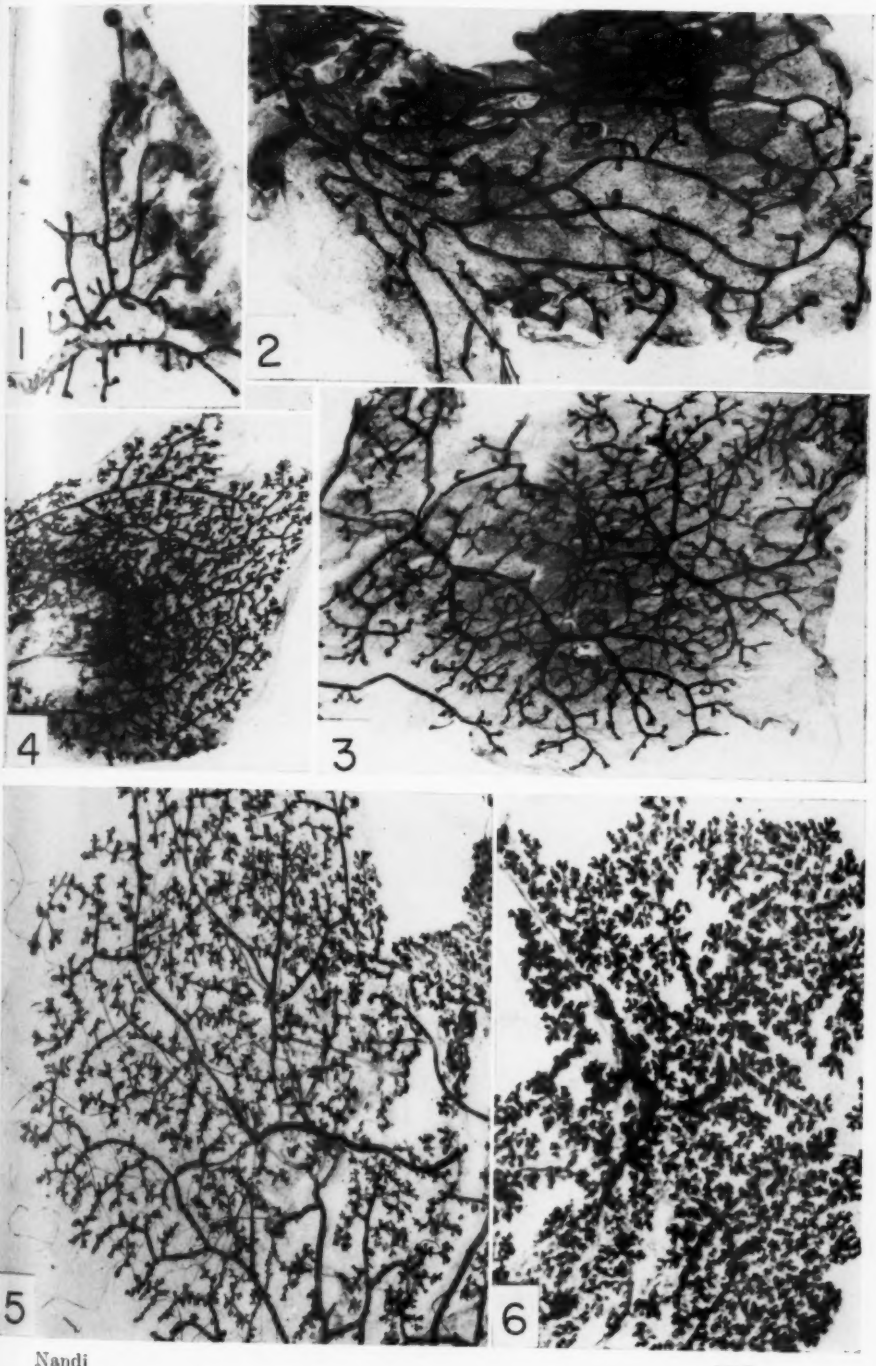

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PLATE 108

FIGURE 7.—Three-day postpartum female. Alveoli are greatly enlarged.

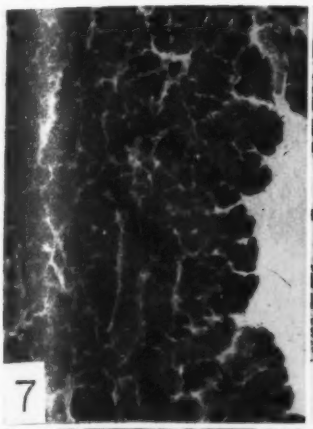
FIGURE 8.—Sections through part of the mammary gland shown in figure 7. *Note* the presence of milk in the alveolar lumina which are delimited by vacuolated epithelial cells.

FIGURE 9.—Twelve-day postpartum female. Individual alveoli are larger than those in figure 8 and the alveolar cells are less vacuolated.

FIGURE 10.—Doubly operated female, 1-month postoperative. *Note* the absence of terminal buds and alveoli.

FIGURE 11.—Triply operated female, 1-month postoperative. *Note* that there is less duct branching than in figure 10.

FIGURE 12.—Triply operated female treated with E (1 μg.) + DCA (200 μg.) + STH (0.5 mg.) daily for 20 days. Stimulation of duct branching, along with the formation of a few terminal buds and alveoli, has occurred.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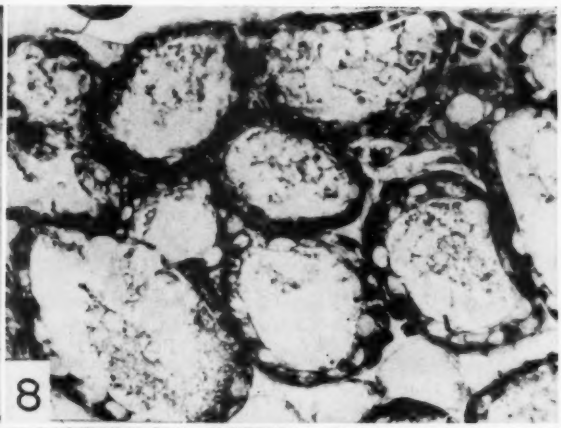
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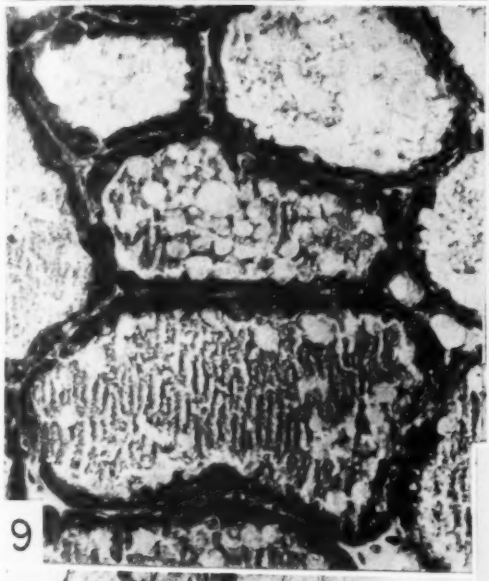
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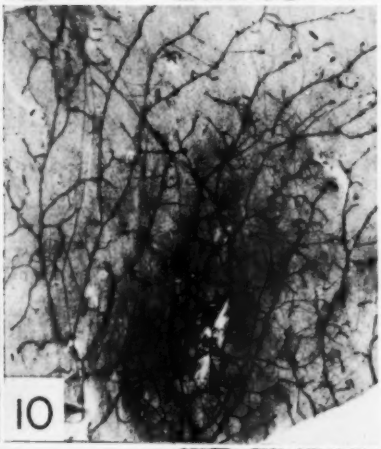
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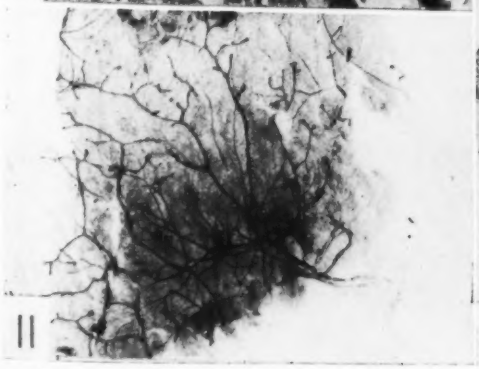
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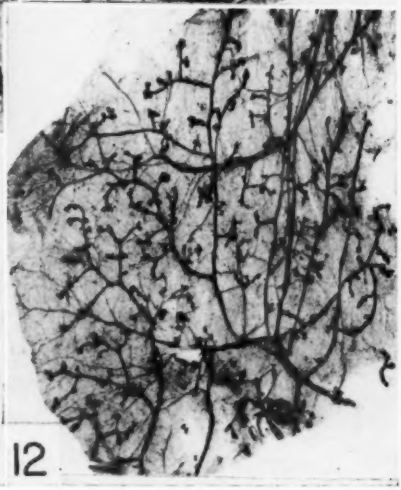
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PLATE 109

FIGURE 13.—Triply operated female treated daily with E (1 μg.) + DCA (200 μg.) + F (62.5 μg.) + STH (0.5 mg.) for 20 days. *Note* that there is more duct branching that in figure 12.

FIGURE 14.—Doubly operated female treated daily with E (2.5 μg.) + P (1 mg.) + DCA (500 mg.) for 30 days. *Note* that the gland contains a few alveoli or alveolar buds.

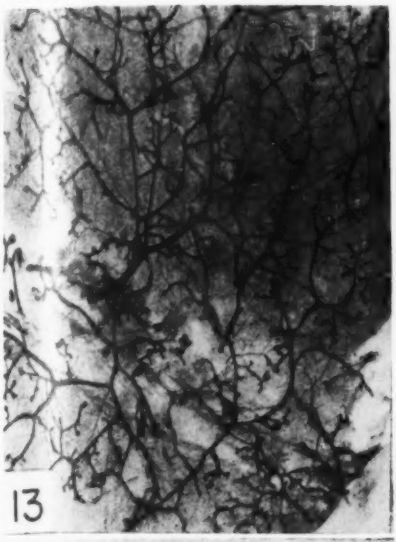
FIGURE 15.—Doubly operated mouse treated daily with E (1 μg.) + P (1 mg.) + MH (0.1 mg.) for 30 days. The mammary development is similar to that shown in figure 4.

FIGURE 16.—Doubly operated female treated daily with E (1 μg.) + P (1 mg.) + STH (0.1 mg.) for 30 days. *Note* the similarity to figure 15.

FIGURE 17.—Triply operated mouse treated daily with E (2.5 μg.) + P (1 mg.) + DCA (500 μg.) + STH (1 mg.) for 30 days. The duct branching is highly stimulated, and large numbers of alveoli have formed.

FIGURE 18.—Doubly operated mouse treated daily with E (2.5 μg.) + P (1 mg.) + STH (1 mg.) for 30 days. The mammary gland contains large numbers of lobules of alveoli.

FIGURE 19.—Doubly operated mouse treated daily with E (2.5 μg.) + P (1 mg.) + MH (1 mg.) for 30 days. *Note* that this mammary gland contains many small lobules.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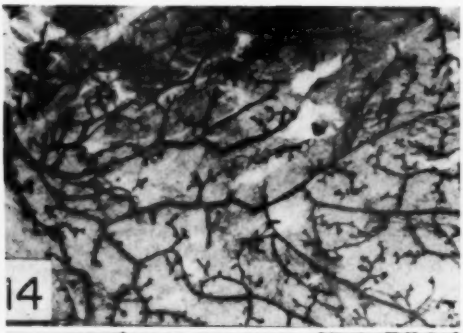
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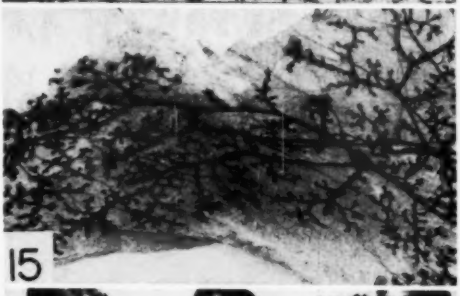
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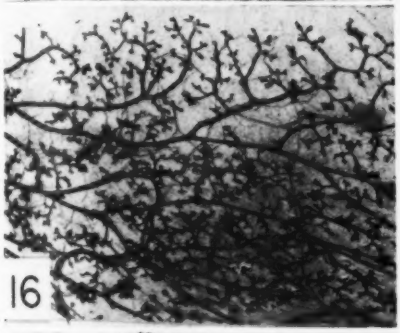
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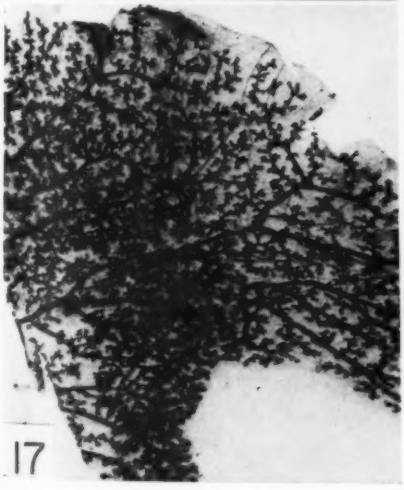
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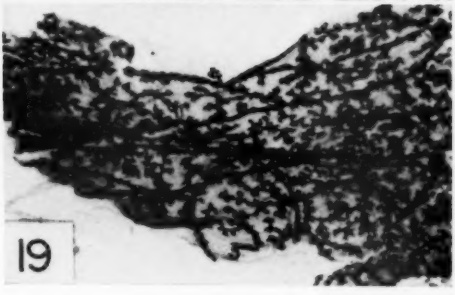
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PLATE 110

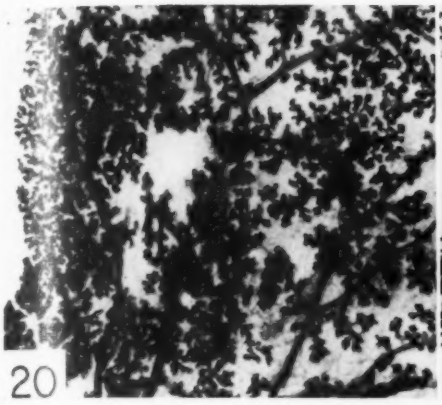
FIGURE 20.—Triply operated mouse treated daily with E (1 μg.) + P (1 mg.) + DCA (500 μg.) + STH (1 mg.) + MH (1 mg.) for 30 days. *Note* the similarity to figure 6.

FIGURE 21.—Triply operated female treated daily with E (1 μg.) + P (1 mg.) + DCA (500 μg.) + F (125 μg.) + STH (1 mg.) for 30 days. This mammary gland contains many well-developed lobules of alveoli.

FIGURE 22.—Triply operated female treated with E (1 μg.) + P (1 mg.) + MH (1 mg.) daily for the first 15 days, followed by MH (1 mg.) + F (125 μg.) for 5 days. The fully developed lobules are filled with milk.

FIGURE 23.—Triply operated female treated with E (1 μg.) + P (1 mg.) + STH (1 mg.) daily for the first 15 days, followed by STH (1 mg.) + F (125 μg.) for 5 days. The lobules are well developed and full of milk.

FIGURE 24.—Triply operated mouse treated with E (1 μg.) + P (1 mg.) + F (62.5 μg.) + STH (1 mg.) + MH (1 mg.) for the first 12 days, followed by MH (1 mg.) + STH (1 mg.) + F (125 μg.) for 5 days. Milk secretion was slightly better than in the mammary glands shown in figures 22 and 23.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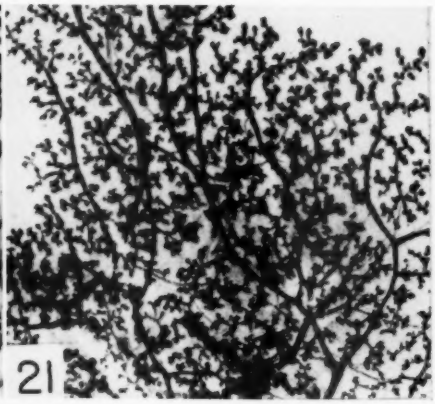
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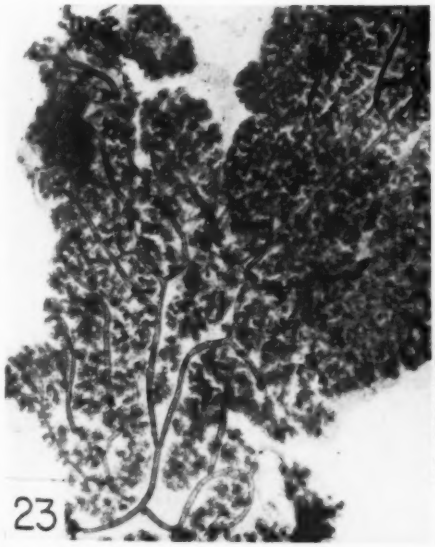
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Influence of Age and of Iodine Intake on the Production of Thyroid Tumors in the Rat [1,2]

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Summary

The influence of age and of iodine intake on the production of neoplastic thyroid nodules was investigated in 1-, 4-, and 14-month-old rats fed for 12 months on a low iodine diet with or without iodine supplement. After autopsy, the number and size of thyroid nodules was estimated in semi-serial sections of the entire gland. While the thyroid glands of the animals given iodine supplement were grossly and histologically normal, those of iodine-deficient rats showed gross enlargement, hyperplasia, decreased colloid, and hyperemia. Nodules were observed and classified on the basis of histological appearance into two broad categories referred to as type-β and type-γ. Type-β nodules were found in 84 of the 135 iodine-deficient rats but in none of the 38 iodine-supplemented rats. Hence the formation of these nodules required the iodine deficiency and, presumably, the high level of circulating thyrotrophic hormone induced by this condition. The proportion of rats with type-β nodules, 74 percent, 52 percent, and 64 percent in the 1-, 4-, and 14-month groups, respectively, was not increased by aging. Type-γ nodules were found in 3 of the iodine-supplemented rats and in 30 of the iodine-deficient rats. Hence iodine deficiency and presumably the resulting high level of circulating thyrotrophic hormone were not essential to, but facilitated the formation of, type-γ nodules. The incidence, 9 percent, 20 percent, and 42 percent in the 3 age groups, respectively, and the size of type-γ nodules increased with age.—**J. Nat. Cancer Inst. 21: 1065–1081, 1958.**

Thyroid glands of rats that were fed on a low iodine diet for long periods were found to contain structures differing from the thyroid tissue, and considered to be neoplastic (*1*, *2*). These structures will be referred to as thyroid nodules. It was further observed that the longer the period of iodine deficiency, the more numerous the nodules (*1*), and the follow-

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ing question arose: Was this increased incidence of thyroid nodules due to the increase in the duration of iodine deficiency or to the fact that the animals were growing older, or to both? The problem was approached by subjecting rats of three different ages to the same period of iodine deficiency. Since controls were given the deficient diet with iodine supplements, it was also possible to investigate the influence of iodine intake on the formation of thyroid nodules.

Materials and Methods

Three groups of female Sherman rats were used. The first or *1-month group* was composed of 104 animals about 1 month old (average body weight 57.6 gm.); the second or *4-month group*, 101 animals 115 to 130 days old (average body weight 177.4 gm.); and the third or *14-month group*, 111 animals 13.5 to 14.5 months old (average body weight 250.4 gm.). The animals were kept 2 to 3 per cage in metal cages with wire-mesh floors. The daily minimum and maximum temperatures of the animal room averaged 76.7 and 85.5° F., respectively, during the experiment.

The diet consisted of 70 percent corn meal, 16 percent wheat gluten, 10 percent yeast, 2 percent extracted liver residue, 1 percent calcium carbonate, and 1 percent sodium chloride. The iodine content estimated according to a modification of the Chaney method (*3*) ranged from 2.9 to 7.1, and averaged 4.6 μg. iodine per 100 gm., thus providing the animals with about 0.5 μg. iodine daily, which is only one fourth or less the amount of the dose of thyroxin required to prevent goiter in thiouracil-treated rats (*4*, *5*).

In each age group three fourths of the rats received distilled drinking water, and one fourth (controls) received distilled water containing 2 μg. iodine per ml. in the form of sodium iodide. A few animals were killed after 155 and 282 days. The 173 remaining rats were killed between the 371st and 380th day. The trachea with the thyroid attached was removed and placed in Bouin's fluid for fixation. After the gland was embedded in paraffin, it was sectioned serially (6 μ) and each 50th section was stained with Masson's light-green trichrome. The approximate volume of each nodule (assumed to be an ellipsoid) was calculated from the diameters in 3 dimensions. Two of the diameters were obtained from the measurements made in each section and the third from the number of sections containing the nodule (the distance between two sections examined was 300 μ). In addition, a few sections taken at random in the gland were stained with hematoxylin and eosin, periodic acid-Schiff, or periodic acid-Schiff and hematoxylin. Kidney, liver, adrenals, heart, spleen, ovaries, and uterus were placed in Bouin's fixative and weighed after 24 hours. The pituitary gland was weighed after fixation in Susa's mixture.

A few days before the rats were killed, the food intake was measured by weighing the food jars at 24-hour intervals.

Results

The thyroid glands of all rats receiving iodine supplements were grossly normal in size. Histologically they consisted of follicles containing moderate amounts of well-stained, nonvacuolated colloid (fig. 1). Follicular cells and" light cells" were seen (fig. 1), as described previously (*1*, *2*).

Animals on the low iodine diet appeared as healthy as those receiving the iodine supplement and their food intake was not significantly different (table 1). The slight increase in body weight caused by iodine deficiency (table 1) was probably due to fat accumulation, since, at autopsy, subcutaneous and peritoneal fat was abundant in iodine-deficient animals but not in the iodine-fed controls. Under the influence of the deficiency, the pituitary weight was increased in the 1- and 4-month groups; liver and kidney weights were diminished in the 1-month group (table 1); adrenal, heart, spleen, ovary, and uterus weights were not modified and therefore are not reported in the table.

The changes observed in the thyroid gland of rats fed the unsupplemented low iodine diet were similar to the changes previously described (*1*): The thyroid glands were grossly very much enlarged and hyperemic; those appearing largest were in the 1-month group. Histologically, the glands of all rats in the 3 groups were the seat of a diffuse hypertrophy and hyperplasia with hyperemia (fig. 2), the enlarged cells being referred to as type-α (*1*).

Many thyroid glands contained solitary or multiple nodules differing architecturally or cytologically from the rest of the gland. The nodules displayed a great diversity of structure but could be classified on a cytologic basis into 2 broad groups, type-β and type-γ (*1*, *2*). Type-β nodules are made up of epithelial, polarized cells, with their bases resting on a membrane and their apexes in contact with a lumen (figs. 3 through 6), and type-γ nodules consist of solid, lobulated masses of lightly basophilic, polygonal cells that show no distinct relation to basement membranes or lumina (figs. 7 and 8).

Although it is possible to subdivide type-β and type-γ nodules (*1*), this was not done in the present work, since the existence of intermediate types between the subcategories would have made quantification unreliable. Furthermore, a small number of nodules already described (*1*) could not be classified as either type-β or type-γ nodules and were omitted from the data.

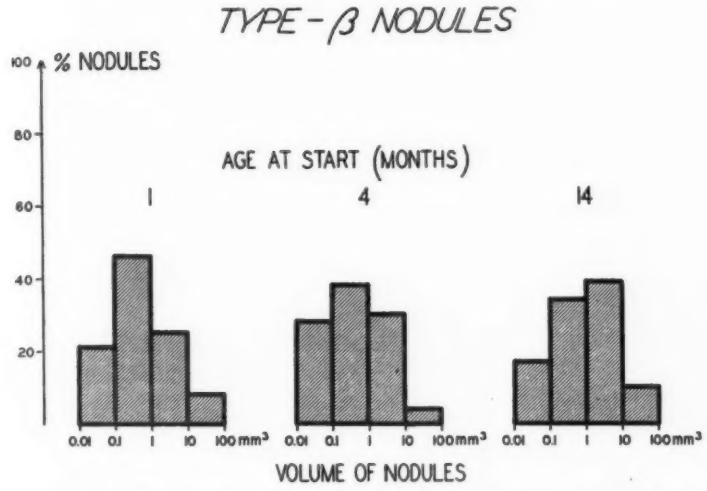
Type-β Nodules

Solitary type-β nodules were found in the thyroid gland of 2 of the 6 rats examined after 155 days and of 2 of the 12 rats examined after 282 days on the low iodine diet. After 371 to 380 days, 84 of 135 animals (62%) had type-β nodules, whereas the 38 rats fed the diet supplemented with iodine had none (table 2). Thus, the presence of 2 μg. iodine per ml. in drinking water prevented the development of these nodules. In order of increasing age, the proportion of the iodine-deficient rats with type-β nodules was 74 percent, 52 percent, and 64 percent (table 3). The

TABLE 1.—*Effect of age and of iodine intake on body weight, weight of pituitary, kidney, and liver, and on food consumption of rats fed a low iodine diet for 12 months*

Age at start of experiment (*months*)	Iodine supplement (*μg. iodine/ml. drinking water*)	Final No. of rats	Body weight (*gm.*)				Pituitary weight (*mg.*)			Kidney weight (*gm.*)			Liver weight (*gm.*)			(Food consumption (*gm./rat/day*)		
			Initial	Final														
			Mean	Mean	S.E.*	*P*	Mean	S.E.*	*P*	Mean	S.E.*	*P*	Mean	S.E.*	*P*	Mean	S.E.*	*P*
1	0	43	58	280	6	0.2	16.2	0.6	0.02	0.89	0.02	0.001–0.01	8.0	0.28	0.01	7.6	0.3	0.05–0.1
	2	10	57	261	12		12.9	1.5		1.15	0.08		9.8	0.59		8.8	0.6	
4	0	56	178	371	8	0.02–0.05	20.7	1.3	0.001–0.01	1.08	0.03	0.7–0.8	11.0	0.33	0.6–0.7	10.1	0.4	0.9
	2	19	177	335	15		15.7	1.0		1.06	0.03		11.3	0.58		10.2	0.6	
14	0	36	252	417	14	0.8	25.3	3.5	0.2	1.29	0.04	0.3	14.0	1.61	0.8–0.9	11.5	0.5	0.8–0.9
	2	9	247	410	27		20.1	2.1		1.41	0.11		14.4	1.42		11.3	0.8	

*Standard error of the mean.



TEXT-FIGURE 1.—Size distribution of type-β nodules in 1-, 4-, and 14-month iodine-deficient rats. Each column corresponds to the nodules falling within 1 of 4 size ranges between 0.01 and 100 mm³. The height indicates the proportion of nodules belonging to each size range. Age had no effect on the size of type-β nodules.

differences were not significant, except in the 1-month as compared to the 4-month group. The size distribution of type-β nodules (text-fig. 1) was not significantly changed by age.

TABLE 2.—*Effect of iodine intake on incidence of type-β thyroid nodules in rats fed a low iodine diet for 12 months*

Iodine supplement (*μg. iodine/ml. drinking water*)	Number of rats	Rats with type-β nodules	
		Number	Percent
0	135	84	62
2	38	0	0

TABLE 3.—*Effect of age on incidence of type-β thyroid nodules in rats fed a low iodine diet for 12 months*

Age at start of expt. (*months*)	Number of rats	Rats with type-β nodules			Total number of type-β nodules
		Number	Percent	*P* against 1-month group	
1	43	32	74	—	75
4	56	29	52	0.02	68
14	36	23	64	0.3	44

Type-γ Nodules

After 155 days on the low iodine diet, none of the 6 rats examined had type-γ nodules in their thyroid gland, but 3 of the 12 rats examined had

type-γ nodules after 282 days. After 371 to 380 days, 30 of 135 iodine-deficient animals (22%) had type-γ nodules. Three of 38 rats (8%) receiving iodine supplements also had such nodules. Thus, type-γ nodules can develop under conditions of adequate iodine intake (fig. 9). However, their incidence was significantly increased by iodine deficiency (table 4), and, furthermore, the size of these nodules was larger in iodine-deficient rats. While the 3 nodules in iodine-fed rats measured less than 0.1 mm.^{3}, 8 of the 35 nodules in iodine-deficient rats measured between 0.1 and 100 mm.^{3}

TABLE 4.—*Effect of iodine intake on incidence of type-γ thyroid nodules in rats fed a low iodine diet for 12 months*

Iodine supplement (*μg. iodine/ml. drinking water*)	Number of rats	Rats with type-γ nodules		
		Number	Percent	*P*
0	135	30	22	0.05
2	38	3	8	

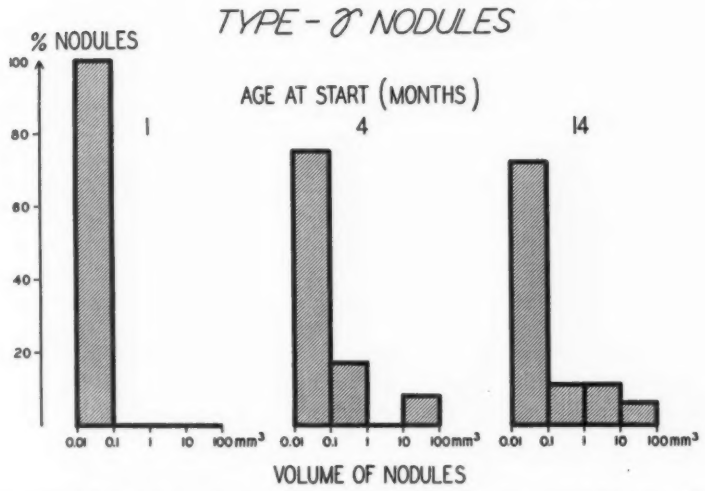
In order of increasing age, the proportion of iodine-deficient animals having type-γ nodules was 9 percent, 20 percent, and 42 percent. The differences between the 14-month group and either of the 2 younger groups were significant (table 5).

With increasing age, there was a shift toward larger nodules in iodine-deficient rats (text-fig. 2), but the differences were not statistically significant.

When type-β and type-γ were compared, the over-all incidence (22%) of type-γ nodules in iodine-deficient rats (table 4) was significantly less ($P < 0.001$) than that of type-β nodules (62%, table 2). The same difference was observed when the 2 types of nodules were compared in the 1- or the 4-month groups only (9% *vs.* 74% and 20% *vs.* 52%; $P < 0.001$), but not when they were compared in the 14-month group (42% *vs.* 64%; $0.1 < P < 0.2$; tables 3 and 5). The size distribution of

TABLE 5.—*Effect of age on incidence of type-γ thyroid nodules in rats fed a low iodine diet for 12 months*

Iodine supplement (*μg. iodine/ml. drinking water*)	Age at start of expt. (*months*)	Number of rats	Rats with type-γ nodules			Total number of type-γ nodules
			Number	Percent	*P* against 1-month group	
0	1	43	4	9	—	5
	4	56	11	20	0.1–0.2	12
	14	36	15	42	<0.001	18
2	1	10	0	0	—	0
	4	19	1	5	0.3–0.5	1
	14	9	2	22	0.3	2



TEXT-FIGURE 2.—Size distribution of type-γ nodules in the 1-, 4-, and 14-month iodine-deficient groups. Arrangement as in text-figure 1. The size of type-γ nodules tends to be greater when age increases.

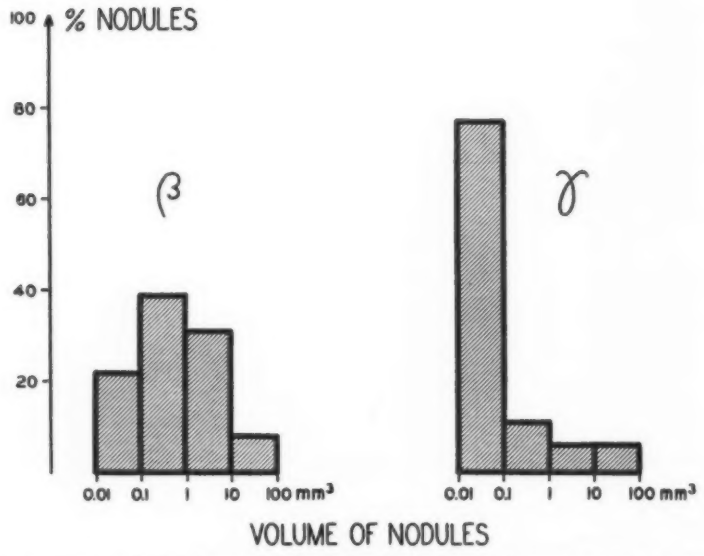
the two types of tumors were significantly different, type-γ being smaller than type-β nodules (text-fig. 3).

Discussion

Recent measurement of thyroid hormone and thyrotrophic hormone in the blood of rats kept for one year on the low iodine diet revealed a very low level of thyroid hormone, 0 to 0.008 μg. protein-bound iodine per ml. plasma (*6*), and a very high level of thyrotrophic hormone (*7*) [estimated according to the method of del Conte (*8*)]. When the diets of the rats were subsequently supplemented with iodine, the protein-bound iodine rose to reach a plateau of about 0.04 μg. protein-bound iodine per ml. after 8 days (*6*), while the level of thyrotrophic hormone decreased in 16 days to less than 1 percent of the value before iodine administration (*7*). Thus there was a low level of circulating thyroid hormone in the iodine-deficient rats. This condition accounts for the increase in pituitary weight and the decrease in kidney and liver weights observed (table 1), since similar, although more pronounced, changes were noted in thyroidectomized animals (*9*).

The over-all sequence of events seems to be that iodine deficiency produces a low level of circulating thyroid hormone, followed by a stimulation of the hypophysis and an overproduction of thyrotrophic hormone, which in turn cause the diffuse thyroid hyperplasia observed in iodine-deficient animals. The larger goiters observed in the 1-month group are in keeping with other evidence (*4*) that younger animals require relatively more thyroid hormone than older ones.

Type-β nodules.—Since type-β nodules were found in iodine-deficient



TEXT-FIGURE 3.—Over-all size distribution of type-β and of type-γ nodules in iodine-deficient rats, obtained by pooling the data for the 3 age groups. The 2 distributions are significantly different, type-β being larger than type-γ nodules.

rats but not in the 38 iodine-supplemented animals (table 2), it appears that under the conditions of the experiment iodine deficiency was a cause of these nodules. Presumably, the high level of thyrotrophic hormone is responsible for their production. Indeed, prolonged administration of thiourea-like compounds, which is also known to result in an oversecretion of thyrotrophic hormone (*10*), has been shown to induce type-β nodules in the thyroid (*11–14*). Furthermore, since a short period of iodine deficiency induces only diffuse hyperplasia, it appears that the thyrotrophic overstimulation must last for a minimal period, 3 to 5 months in iodine-deficient rats, before type-β nodules appear (*2*).

The incidence of type-β nodules did not increase with age (table 3). It thus appears that the larger number of these nodules observed when the iodine deficiency period was increased (*1*) was not due to the more advanced age of the rats but to the longer period of thyroid overstimulation. The slight but significantly higher incidence observed in the youngest rats (table 3) is probably due to the fact that the thyrotrophic overstimulation was more intense in these animals, as indicated by larger goiters than in the older groups.

In conclusion, the production of type-β nodules appears to depend on the intensity and duration of thyrotrophic overstimulation, but does not seem to be increased by age.

Type-γ nodules.—The cells making up type-γ nodules (fig. 7) are similar to light cells (fig. 1), and evidence has been presented that these cells probably give rise to the nodules (*1*, *2*). Indeed, the question arose as

to whether the usually small type-γ nodules seen in iodine-fed rats were not mere aggregations of light cells. However, the large size of one nodule (fig. 9) and the invasive properties of another (*1*) demonstrated that characteristic type-γ nodules could arise in rats fed on an adequate iodine intake.

Since iodine deficiency increased the incidence (table 4) and probably the size of type-γ nodules and since these nodules had often been observed in animals treated with thiourea-like compounds (*13*, *15*, *16*), it is likely that a high level of thyrotrophic hormone stimulates their formation. However, the few nodules observed in animals on an iodine-supplemented diet (table 4) indicate that a normal level of this hormone is sufficient to induce type-γ nodule formation to some extent. If overstimulation with thyrotrophic hormone, though not essential, facilitates the occurrence of type-γ nodules, then an increased duration of this overstimulation would result in more nodules. Nevertheless, aging may also increase the incidence of type-γ nodules (table 5). Thus the increased number of type-γ nodules observed when the duration of iodine deficiency was increased (*1*) was due to both the longer duration of thyroid overstimulation and aging.

It may be objected that the lower incidence of type-γ nodules in the 1-month group as compared to the older groups (table 5) might result from the fact that 1) the 1-month group had a higher proportion of small nodules than the older groups (text-fig. 2), and 2) since nodules are counted in every 50th section only, small nodules may be missed. As a result, more nodules would be overlooked in the 1-month group than in the older groups. However, the difference in size distribution between young and adult rats is probably too small to account for the large difference in nodule incidence (text-fig. 2). Moreover, when only those type-γ nodules small enough to be missed were considered, that is, those found in one section only, their number did not decrease with age as expected if the objection were valid, but *increased* from 7 per 100 rats in the 1-month group to 25 per 100 rats in the 14-month group.

It is a common belief that the incidence of tumors in general increases with age. In the case of the thyroid gland this belief has been substantiated by Krook (*17*), who found that the average age of 808 dogs which he autopsied was 4.3 years, while that of 98 dogs in which he found thyroid carcinoma was 9.9 years. Many hypotheses have been proposed to explain the increased incidence of tumor with age. Most of them derive from the observation that the rate of death from cancer in man increases proportionately to the logarithm of age (*18–22*), but these hypotheses do not fit with our data. In the present experiment the relationship between the incidence of type-γ nodules and age approximated a straight line. Such a relationship may be explained in terms of the "two-phase" hypothesis for tumor formation (*23*) as applied by Hall (*24*) to the thyroid gland. During a first or initiation phase, normal cells would be transformed into latent neoplastic cells, an effect assumed to be produced by a carcinogen. During the second or promoting phase,

the latent neoplastic cells would develop into a tumor, a process presumably influenced by mitogenic agents. Since carcinogens were not used for the production of the nodules described in the present work, the hypothesis of Hall (*24*) must be broadened to include the possibility that during the initiation phase normal thyroid cells would be transformed into latent neoplastic cells spontaneously and at random, so that in time the latter cells would accumulate in the thyroid. Growth of these cells to form visible nodules would be promoted by thyrotrophic stimulation.

According to this hypothesis, the thyroid gland of the animals that were older at the beginning of the treatment would be expected to contain more latent neoplastic cells and, after the period of iodine deficiency, show more nodules. Furthermore, if the initiation phase for each nodule consisted of a single event, then a straight-line relationship would be expected between incidence of nodules and age. Thus, aging would influence the initiation phase by increasing the number of latent neoplastic cells, while iodine deficiency would influence the promoting phase by shortening the time required for the latent cell to grow into a visible nodule.

This hypothesis fits the observations made on type-γ nodules, but does not apply to type-β nodules. The different effects of age and iodine deficiency on the induction of type-β and type-γ nodules suggest that different mechanisms are involved in their formation.

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PLATES

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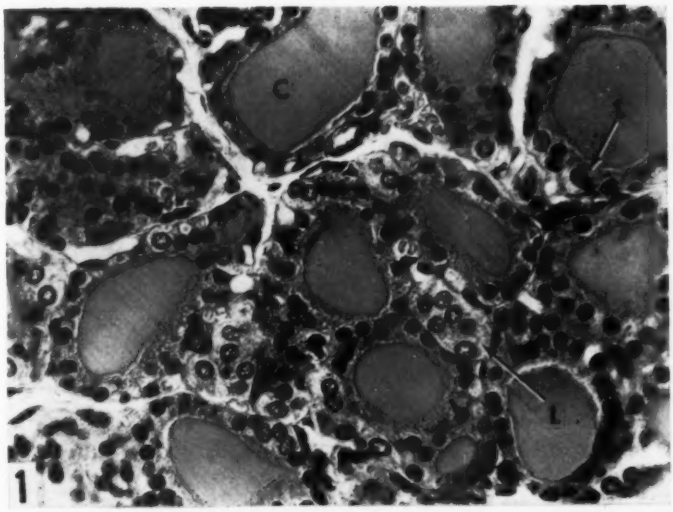
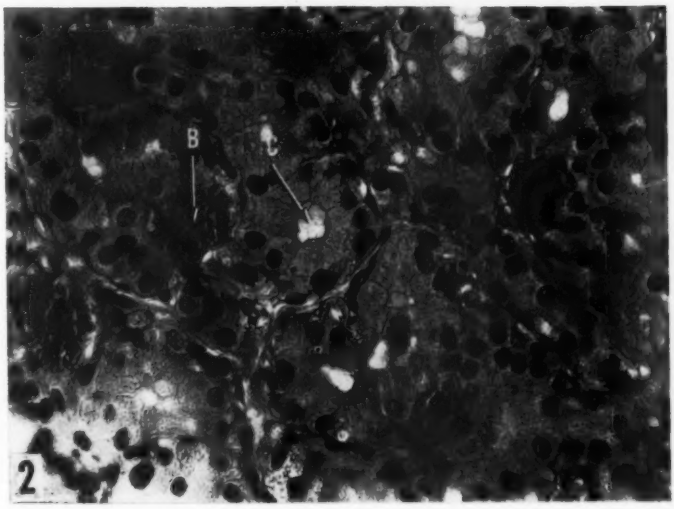
PLATE 111

Normal thyroid tissue of rats fed for 12 months on the low iodine diet supplemented with iodine (fig. 1) or unsupplemented (fig. 2). Hematoxylin and eosin. × 410

FIGURE 1.—In iodine-supplemented rats the amount of colloid (C) is large, and the light cells (L) with large, vacuolated nucleus and pale cytoplasm are easily distinguished from the follicular cells with dark nucleus and well-stained cytoplasm.

FIGURE 2.—In iodine-deficient animals the amount of colloid (C) is scanty, blood vessels (B) are numerous, and light cells are usually not distinguishable.


C
L
1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C
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PLATE 112

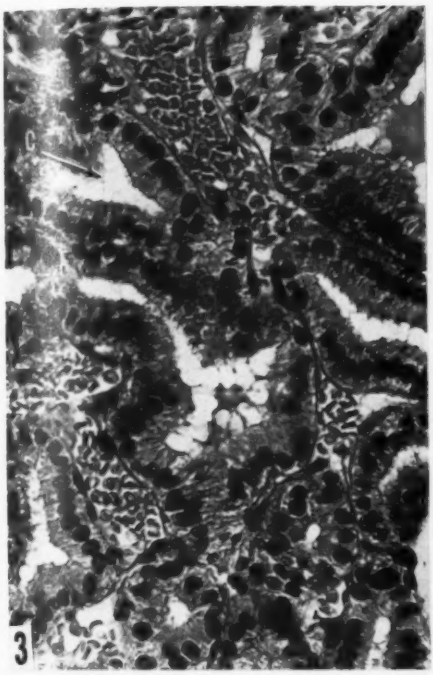
Type-β nodules in thyroids of rats fed for 12 months on the low iodine diet without iodine supplement. A single-layered epithelium is in contact with a lumen which contains a variable amount of colloid-like material (C). The nuclei are located at the base of the cells and in figures 3 through 5 are hyperchromatic. Hematoxylin and eosin. × 410

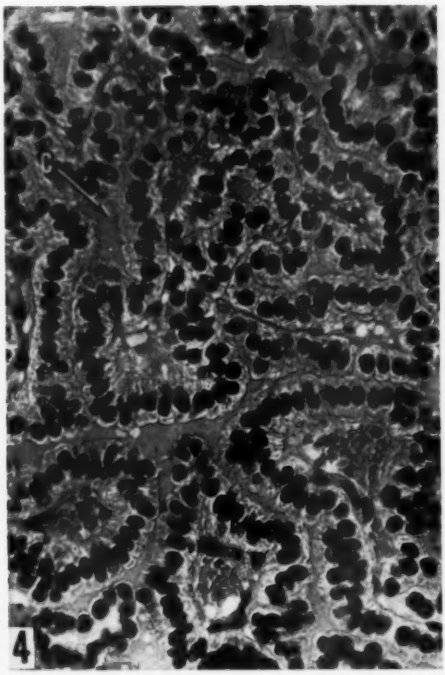
FIGURE 3.—A high columnar epithelium lining convoluted, tube-like follicular structures, previously referred to as type-β_1 (*1*). Colloid may be seen at *upper right* and is often vacuolated (*center*).

FIGURE 4.—A low columnar epithelium with very convoluted follicular structures. Colloid is little or not vacuolated.

FIGURE 5.—A cuboidal epithelium with dense nuclei and scanty cytoplasm in all but a few cells, previously referred to as type-β_2 (*1*).

FIGURE 6.—A type-β nodule differing from the others mainly by the lightly stained and irregularly shaped and sized nuclei and by the presence of several layers of cells. Scattered neutrophils may be seen.


C
3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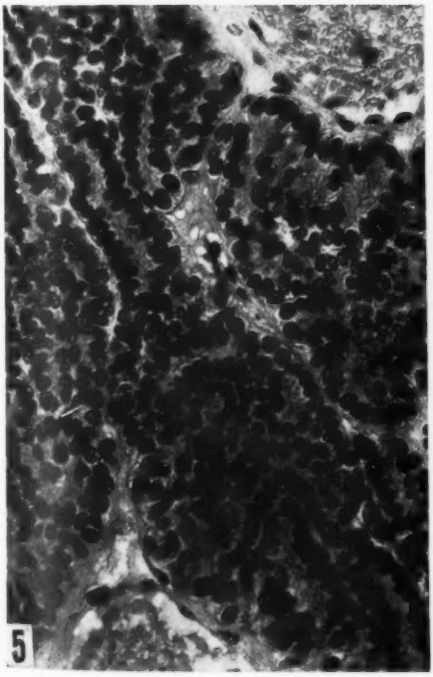
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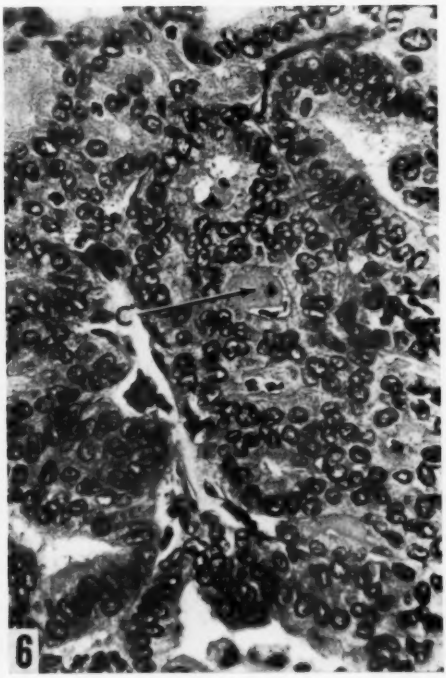
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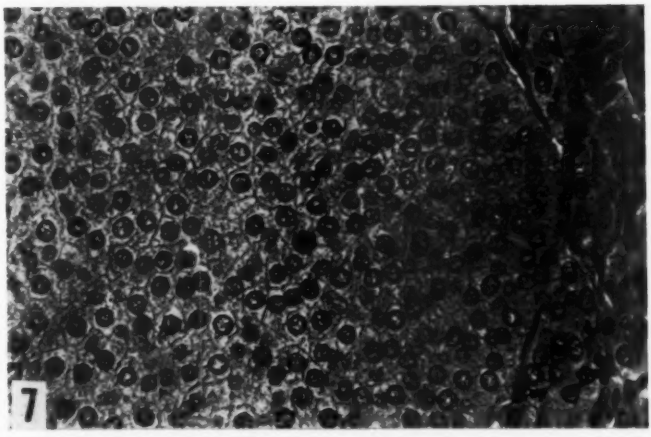
PLATE 113

Type-γ nodules in thyroids of rats fed for 12 months on the low iodine diet without (figs. 7 and 8) and with (fig. 9) iodine supplement.

FIGURE 7.—Normal hyperplastic tissue at extreme *right*, while a solid mass of polygonal type-γ cells occupies the rest of the field. The nuclei are large, oval, and contain finely granular chromatin and often a transparent vacuole. Such a nodule was previously referred to as type-γ_1 (*1*). Masson trichrome. × 410

FIGURE 8.—Densely packed type-γ cells with densely stained nuclei and numerous mitotic figures. Such a nodule was previously referred to as type-γ_2 (*1*). Hematoxylin and eosin. × 410

FIGURE 9.—Type-γ nodule in the thyroid of a rat fed diet supplemented with iodine. The lightly stained nodule stands out among colloid-laden follicles. Periodic acid-Schiff-hematoxylin. × 75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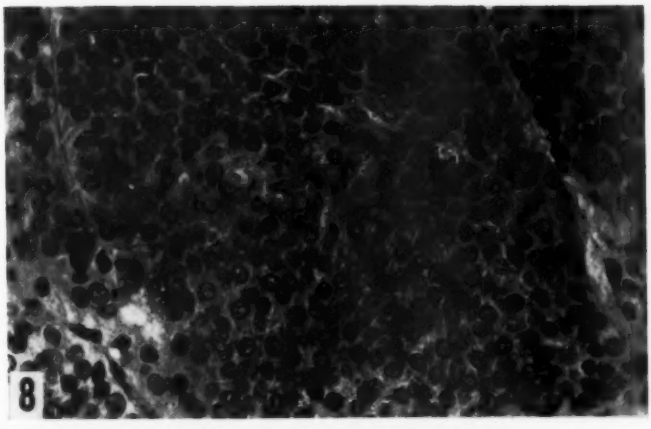
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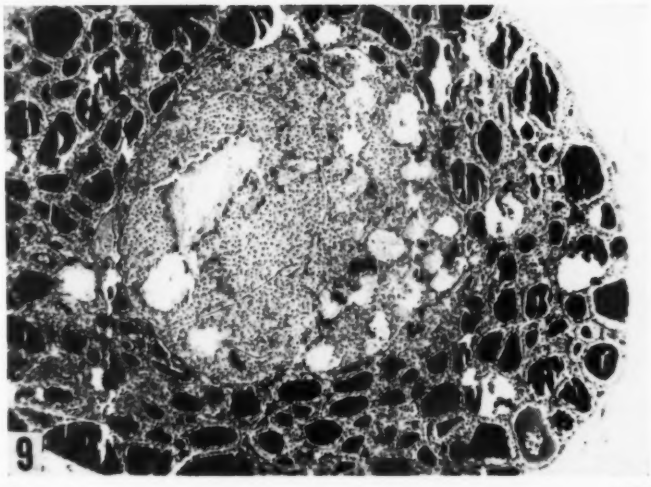
9

Role of Implanted Inbred-Strain Thymic Tissue in the Development of Leukemia in F_1 Hybrid Mice [1,2]

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Summary

Two experiments attempting to elucidate the role of the thymus in the development of leukemia and a third experiment testing for the presence of a leukemogenic virus in leukemic cells were performed. Subcutaneous implantation of thymic tissue from mice of strains C57BL and DBA into (C57BL × DBA)F_1 hybrids and from strain C57BL and C3H mice into (C57BL × C3H)F_1 hybrids was followed in 3 to 4 weeks by injections of 0.02 mg. of 7,12-dimethylbenz[*a*]anthracene into the thymic grafts. No tumors appeared in the thymic grafts, and no influence on the incidence of leukemia in the hosts was observed. Subcutaneous implantation of thymic tissue from DBA/2 mice into (CBA × DBA/2)F_1 hybrid mice showed no effect, whereas implantation of CBA thymic tissue into F_1 hybrids of the same genetic constitution accelerated the development of leukemia in the hosts. No leukemic tumors were found in the thymic grafts. Leukemic tissue from DBA/2 mice was killed by X irradiation *in vivo* and injected intraperitoneally into (CBA × DBA/2)F_1 mice. No influence on the incidence of leukemia was observed. In experiments 2 and 3, the incidence of leukemia in 213 (CBA × DBA/2)F_1 mice over 18 months of age was about 50 percent. Most of the leukemias were of the plasma-cell variety; several of them and their transplantation lines showed abnormal serum-protein fractions in the β- or γ-globulin ranges. Several reticulosarcomatoses and localized reticulosarcomas, one atypical, presumably monocytic leukemia, and a number of leukemias, probably lymphatic, were observed.—**J. Nat. Cancer Inst. 21: 1083–1098, 1958.**

In 1944 McEndy *et al.* (*1*) showed that thymectomy strikingly reduced the incidence of spontaneous leukemia in a high-leukemic strain of mice (AK), whereas splenectomy did not alter the incidence. This finding was soon confirmed in spontaneous (*2*, *3*), carcinogen-induced (*4*), and radiation-induced leukemia (*5*). It has been demonstrated also that subcutaneous implantation of thymic grafts into thymectomized mice

[1] Received for publication June 9, 1958.

[2] These investigations were supported by grants from Anders Hasselbalch's Fund, The King Christian X Fund, and The Leukemia Society, Inc., New York, N. Y.

[3] When the experimental part of these investigations was performed, the author held a part-time scholarship from The Lady Tata Memorial Fund from 1952–55.

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wholly or partially prevented a reduction in the incidence of carcinogen-induced (*4*) and radiation-induced (*6*) leukemia caused by thymectomy.

The ability of the thymus to transform normal cells into leukemic cells has been genetically determined. Lymphocytic neoplasms have been found to develop in the thymus of strain C57BL (*7*) and AKR (*8*) mice, but not in C3H thymic tissue (*7*, *8*) implanted subcutaneously into F_1 hybrids between the parent strains.

Factors in the host organism, however, also exert a certain influence on thymic transformation. It has been found that leukemia developed in nonirradiated thymic grafts implanted subcutaneously into irradiated, thymectomized C57BL mice (*9*), but did not develop in the implanted thymus of nonirradiated C57BL mice (*10*).

Reversely, the implanted thymus may exert a certain leukemogenic effect on the host organism. Thus, the incidence of F_1 leukemias increased considerably after implantation of thymic tissue from a high-leukemic strain (RIL) into F_1 hybrid mice between this strain and a low-leukemic strain (*11*). It has been found also (*12*) that implantation of thymic tissue from C57BL mice into thymectomized, irradiated (C57BL $\times$ A)F_1 mice increased the incidence of leukemia. The earliest leukemias that developed consisted of C57BL cells and the later ones of F_1 cells, as estimated from histocompatibility tests.

In addition to these investigations, a series of experiments from this laboratory indicated the exceptional role of the thymus in the development of leukemia. Thus, injection of a very small dose (0.02 mg.) of 7, 12-dimethylbenz[*a*]anthracene[4] directly into the thymus (*13*, *14*) induced leukemic thymic tumors or generalized leukemia in 13 percent of ST mice, in 19 percent of DBA mice, in 13 percent of C57BL mice, but in only 2 percent of C3H mice. The spontaneous incidence of leukemia in these strains reaches 1 percent. Direct injection of the same dose of this hydrocarbon into other tissues (lymph nodes, spleen, bone marrow, liver, kidney, testis, subcutaneous tissue, skin) did not induce leukemia; even by direct injection of a very large dose (0.5 mg.) of 7,12-dimethylbenz[*a*]-anthracene, it proved impossible to induce leukemic tumors in the lymph nodes and spleen of ST mice (*13*). Furthermore, when 0.02 mg. doses of dibenzanthracene and methylcholanthrene were injected into the thymus of ST mice (*15*), lymphosarcomas developed in 6.7 and 10.4 percent, respectively, whereas injection of the same dose of benzopyrene had no effect.

Thus the thymus is a most essential factor in the development of leukemia in mice, not only as the primary site of formation of the malignant leukemic cells but also in some other, still unexplained, way.

A description will be given of two experiments performed to elucidate further the role of the thymus in the development of leukemia. A third experiment, designed to test for a leukemogenic virus in leukemic cells, is included so that the development of neoplasias of the reticular tissue observed in old (CBA $\times$ DBA/2)F_1 hybrid mice of the second and third experiment may be reported jointly.

[4] Former designation: 9,10-dimethyl-1,2-benzanthracene.

Experiment 1

In this experiment, thymuses from two strains, DBA and C57BL, in which the thymus had proved susceptible to direct injection of 0.02 mg. of 7,12-dimethylbenz[*a*]anthracene, and the thymuses from strain C3H, in which the thymus had proved refractory to such injection (*14*), were implanted subcutaneously into F_1 hybrids between the strain of the implanted thymus and one of the other strains. After the implanted thymuses had completely regenerated, within 3 to 4 weeks (*16*), 0.02 mg. of 7,12-dimethylbenz[*a*]anthracene was injected directly into them.

By this experimental design it was possible to observe whether the carcinogenic action would induce thymic tumors in the implanted thymus, to establish by histocompatibility tests whether such thymic tumors consisted of inbred-strain cells, and, furthermore, to observe whether implantation of the thymus of a given genetic constitution would exert any influence on the incidence of F_1 leukemias.

Materials and Methods

The mice used were F_1 hybrids between C57BL and DBA and between C57BL and C3H mice, with the mammary-tumor agent. All mice were bred in this laboratory; the inbred mice by brother × sister mating. When the F_1 mice were 1 to 3 months old, a whole thymus from a mouse, usually 1 to 3 months old, of one of the parent strains was implanted subcutaneously into the right flank near the axilla. Three to 4 weeks later the implanted thymus was inspected through an incision in the skin, and 0.02 mg. of 7,12-dimethylbenz[*a*]anthracene dissolved in 0.01 cc. paraffin was injected directly into the regenerated thymus before the incision was closed. Experimental and control mice were not litter mates.

All mice were allowed to live until spontaneous death occurred, and then autopsied. If any signs of leukemia were found, the lungs, liver, spleen, kidneys, and lymph nodes were fixed in formalin, stained with hematoxylin-eosin, and examined microscopically.

Results

All mice were observed once a week. Tumors were not found in any of the thymic transplants. However, leukemia developed in experimental as well as in control mice, but not until the mice were at least 21 months of age. The occurrence of leukemia is shown in table 1.

The average age (25 to 28 months) of the leukemic mice was similar for all groups.

The incidence of leukemia was the same in mice with DBA thymic grafts and their corresponding (C57BL × DBA)F_1 controls (14 and 18%, respectively) and in mice with C3H grafts and their corresponding (C57BL × C3H)F_1 controls (13 and 14%, respectively). This incidence indicates that implantation of DBA and C3H thymic tissue had no influence on the development of leukemia.

In mice with C57BL thymic grafts, the incidence of leukemia was somewhat higher, viz., 40 percent in (C57BL × DBA)F_1 hybrids and

Table 1.—Incidence of leukemia in experiment 1

Strain	Age of mice (*months*): 21–22	23–24	25–26	27–28	29–30	31–32	33–34	35–36	37–38	Mice (*total No.*)	Incidence of leukemia (*percent*)	Average age (*months*)
(C57BL × DBA)F_1 mice												
With C57BL thymic graft {with leukemia	1		1		1	1				4	40	27. 5
without leukemia	1		1		1	2			1	6		
With DBA thymic graft {with leukemia	1	1			1					3	14	25. 0
without leukemia	6	5	2	4	1	1				19		
Untreated {with leukemia	1	1	2	5	1	2				12	18	27. 2
without leukemia	1	12	7	14	5	2	8	6		55		
(C57BL × C3H)F_1 mice												
With C57BL thymic graft {with leukemia			1	2	2	1				6	21	28. 5
without leukemia	5	2	5	1	3	2	2	2	1	23		
With C3H thymic graft {with leukemia	1		1		1					3	13	25. 3
without leukemia	2	1	3	5	3	3	1		2	20		
Untreated {with leukemia		1	2	1						4	14	25. 7
without leukemia	5	5	3	1	4	1	3	2		24		

21 percent in (C57BL × C3H)F_1 hybrids. The increased incidence in both groups of mice with C57BL thymic grafts indicates that implantation of C57BL thymic tissue probably exerted some leukemogenic effect. However, the number involved is too small to permit definite conclusions.

The thymic transplants could not be recovered at autopsy of the leukemic mice. In a few mice, very small fibrous swellings were found, which, under microscopic examination, consisted of fibrillar connective tissue with little or no thymic tissue. This observation agrees with the previous finding (*16*) that the involution of the thymic graft corresponds to the age of the host.

No attempt was made to transplant the leukemias that arose. It seemed extremely unlikely, because of the advanced age of the leukemic mice and because the implanted thymus was not involved in the leukemic process, that these leukemias consisted of cells of the thymic graft.

Most of the leukemias were of the plasma-cell variety, the grade of differentiation being II to IV, according to the classification in our previous papers (*17*, *18*). A few were best classified as reticulosarcomatoses, 2 were lymphatic, and a few could not be classified because of *post-mortem* changes. Some of the plasma-cell leukemias have been described previously (*18*), and the others did not differ from them. The frequency of gross and microscopic findings is indicated in table 2.

Among the experimental mice as well as among the controls, there were some hepatomas, pulmonary adenomas and carcinomas, a few skin carcinomas and subcutaneous spindle-cell sarcomas, and a fairly large number of mammary carcinomas.

In summarizing this experiment it may be stated that subcutaneous implantation of DBA, C3H, and C57BL thymic tissue, followed by injection of carcinogenic hydrocarbon into the grafts, did not result in the development of leukemic tumors in the implanted thymus. The DBA and C3H grafts had no effect, whereas C57BL grafts possibly had a leukemogenic effect on the host.

Experiment 2

Since leukemic tumors did not develop in the implanted thymus of DBA, C57BL, and C3H origin, a new experimental series was started with CBA or DBA/2 thymic transplants into F_1 hybrid mice. CBA mice are known to develop spontaneous leukemia only rarely, whereas the incidence of leukemia in DBA/2 mice is 30 to 40 percent (*19*). Since it was considered probable that a reasonable number of leukemic tumors would develop in the implanted thymus from a relatively high-leukemic strain like DBA/2, injection of carcinogenic hydrocarbon was omitted in this experiment.

Materials and Methods

The mice used were (CBA × DBA/2)F_1 hybrids, all bred in this laboratory by brother × sister matings. When the mice were 3 to 6 weeks old, a thymic lobe from a CBA or DBA/2 mouse, 1 to 2 months old, was

Table 2.—Gross and microscopic findings in leukemic mice of experiment 1

Strain	Leukemias (*number*)	Examination of lesion	Organ						
			Thymus	Spleen	Liver	Kidneys	Perirenal tissue	Lymph nodes	Lungs
C57BL × DBA mice with C57BL thymic graft	4	Gross	2	2	1		3	4	
		Microscopic	2	3	3	1	2	4	2
C57BL × DBA mice with DBA thymic graft	3	Gross	1		2		2	1	
		Microscopic	1	2	2	1	2	1	
C57BL × DBA mice, untreated	12	Gross	2	6	8		6	7	
		Microscopic	3	8	10	7	6	7	4
C57BL × C3H mice with C57BL thymic graft	6	Gross	1	6	5		2	4	
		Microscopic	4	6	6	2	3	4	2
C57BL × C3H mice with C3H thymic graft	3	Gross		1	2		1	1	
		Microscopic		3	2			1	2
C57BL × C3H mice, untreated	4	Gross	2	3	3		2	4	
		Microscopic	1	3	2	1	4	4	3

implanted subcutaneously into the right axilla. Experimental and control mice were litter mates.

The mice were allowed to live until spontaneous death occurred. All mice were autopsied and when gross inspection revealed leukemic changes, the lungs, liver, spleen, kidneys, and lymph nodes—in some cases also other organs—were removed for microscopic examination.

Results

All mice were inspected once a week. Tumors were not found in any of the thymic transplants. Table 3 shows the incidence of leukemia in these mice.

The incidence of leukemia in the mice with DBA/2 thymic grafts was 36 percent and that of the corresponding controls 48 percent. The average age was 27.2 and 29.4 months, respectively.

Among female animals, 52 percent of those receiving thymic grafts and 15 percent of the control mice developed mammary carcinomas at a mean age of 19.7 and 29.6 months, respectively. The somewhat lower incidence of leukemia among the experimental mice was presumably due to their considerably higher incidence of mammary carcinoma, which acted as a competing cause of death. The increased incidence of mammary carcinoma again was probably due to the implantation of thymic tissue from DBA/2 mice, which carry the mammary-tumor agent. All experimental and control mice had CBA mothers and DBA/2 fathers and thus did not carry the agent themselves.

Implantation of DBA/2 thymic tissue probably had no influence on the development of leukemia.

Table 3 also shows that among mice over 10 months of age carrying CBA thymic grafts, leukemia occurred in 42 percent. Among their littermate controls, leukemia was observed in 22 percent. The mean age was 20.8 and 29.6 months. Considering that the incidence of leukemia in the control group for the mice with DBA/2 thymic grafts was 48 percent, it is perhaps not correct to place too much emphasis on the difference in the incidence of leukemia between mice with CBA thymic grafts (42%) and their controls (22%). However, there is no doubt that *the implantation of the CBA thymus accelerated the development of leukemia.* Table 3 shows that 8 of 14 leukemic experimental mice had died before 26 months when the first of the 8 leukemic control mice died.

The leukemias occurring in F_1 hybrids with CBA thymic grafts did not differ in any respect from the others observed.

In regard to the implanted thymus, the findings were the same as in experiment 1.

Three of the leukemias were transplanted to mice of the inbred strain from which the thymic graft originated, with negative results, and to F_1 hybrid mice, with positive results.

The development of leukemia was analogous to that observed in experiment 3, also performed with (CBA $\times$ DBA/2)F_1 mice. The leukemias in these 2 experiments will therefore be described after experiment 3.

Table 3.—Incidence of leukemia in experiment 2

Strain (CBA × DBA/2)F_1 mice		Age of mice (*months*): 10–11	12–13	14–15	16–17	18–19	20–21	22–23	24–25	26–27	28–29	30–31	32–33	34–35	36–37	Mice (*total No.*)	Incidence of leukemia (*percent*)	Average age (*months*)
With DBA/2 thymic graft	with leukemia							2	2	3	2	1	1	1		12	36	27. 2
	without leukemia							1	3	4	8	1	1	2	1	21		
Untreated	with leukemia								3	2	2	3	3	1		14	48	29. 4
	without leukemia								6	3	2	1	1	2		15		
With CBA thymic graft	with leukemia	1	1	1	2		1		2	1		3	1	1		14	42	20. 8
	without leukemia				1	1	3	4	1	2	1	3	2	1		19		
Untreated	with leukemia									2	1	3	2			8	22	29. 6
	without leukemia		1			3	5	2	2	6		6	3			28		

The frequency of gross and microscopic findings is given in table 5. No division into various types of leukemia is shown. Most of the leukemias observed in the mice carrying CBA thymic grafts were of the plasma-cell variety, two were probably stem-cell leukemias and a few could not be classified.

In conclusion it may be stated that subcutaneous implantation of DBA/2 and CBA thymic tissue was not followed by leukemic tumor formation in the implanted thymus. DBA/2 thymic grafts showed no influence, whereas CBA grafts accelerated the development of leukemia in the host.

Experiment 3

On the basis of Ellermann and Bang's finding (*20*) that leukemia in chickens may be transmitted by ultrafiltrates of leukemic tissue, in 1938 the author performed an experiment with injection of minced leukemic tissue irradiated *in vitro* with 5000 to 10,000 r (*21*).

Malignant cells are killed within a few hours by this dose, whereas viruses are resistant (*22,23*). It is possible that a leukemogenic virus, introduced into the host organism in cells that are still alive, has a better chance of infecting the hematopoietic cells of the host than a cell-free extract of leukemic cells. The experiment was performed on noninbred mice and gave a negative result, with no development of leukemia.

After Gross published his findings in 1951 (*24, 25*), this experiment was repeated with leukemic tissue from DBA/2 mice, which, after X irradiation *in vivo*, was injected into (CBA × DBA/2)F_1 mice.

Materials and Methods

As in experiment 2, the mice used were (CBA × DBA/2)F_1 hybrids, with littermate control animals.

Leukemic tissue was procured from mice carrying subcutaneous transplants from a stem-cell leukemia originally induced by painting DBA/2 mice with 7,12-dimethylbenz[*a*]anthracene. The leukemic mice were irradiated with 4500 to 6000 r.[5] Immediately after irradiation the mice were killed, the leukemic tissue was minced, and 0.05 to 0.1 cc. of the minced tissue was injected intraperitoneally into (CBA × DBA/2)F_1 mice from a few hours to 6 weeks old.

All mice were allowed to live until spontaneous death, and then autopsied. When gross inspection revealed leukemic changes, the lung, liver, spleen, kidney, lymph nodes, and, in some cases, other organs as well, were examined microscopically.

Results

The mice were inspected once a week and where feasible, were killed *in extremis*. The earliest instance of leukemia was seen in a mouse 18 months old.

Table 4 shows that leukemia was found in 63 percent of experimental mice at a mean age of 25.0 months and in 52 percent of control mice at

[5] The irradiations were carried out at the Radium Centre of Copenhagen through the courtesy of its director, Professor Jens Nielsen, M. D.

Table 4.—Incidence of leukemia in experiment 3

Strain (CBA × DBA/2) F_1 mice		Age of mice (*months*): 18–19	20–21	22–23	24–25	26–27	28–29	30–31	32–33	34–35	Mice (*total No.*)	Incidence of leukemia (*percent*)	Average age (*months*)
Injected with radiation-killed leukemic tissue	with leukemia	4	3	1	6	8	8	1			31	63	25. 0
	without leukemia	1	2	3	1	7	2	2			18		
Untreated	with leukemia	1	1	3	4	2	5			1	17	52	25. 6
	without leukemia		2		2	5	4	3			16		

Table 5.—Gross and microscopic findings in leukemic mice of experiments 2 and 3

Strains	Leukemias (*number*)	Examination of lesion	Organ						
			Thymus	Spleen	Liver	Kidneys	Perirenal tissue	Lymph nodes	Lungs
(CBA × DBA/2)F_1 mice with DBA/2 thymic graft.	12	Gross Microscopic	2 2	7 9	1 8	2 6	3 6	9 9	 4
(CBA × DBA/2)F_1, untreated	14	Gross Microscopic	3 6	9 12	2 8	1 9	9 9	9 9	 8
(CBA × DBA/2)F_1 mice with CBA thymic graft.	14	Gross Microscopic	5 5	10 11	6 9	 6	5 8	14 14	 3
(CBA × DBA/2)F_1, untreated	8	Gross Microscopic	3 3	7 7	 5	1 5	4 4	4 6	 1
(CBA × DBA/2)F_1 mice injected with radiation-killed tissue.	31	Gross Microscopic	6 6	16 26	9 26	2 13	18 18	25 26	 5
(CBA × DBA/2)F_1 mice, untreated	17	Gross Microscopic	5 6	8 13	7 11	3 8	8 9	10 12	 3
Total number	96								

a mean age of 25.6 months. The advanced age of the leukemic mice and the similar incidence of leukemia in experimental and control mice show that the injected, irradiated leukemic cells were unable to reproduce. It also is evident that injection of irradiated leukemic tissue had no influence on the development of leukemia. The frequency of gross and microscopic findings is shown in table 5.

Many of the leukemias have been transplanted to F_1 hybrids with positive results; some have also been transplanted to DBA/2 mice, all with negative results.

Character of Leukemias in (CBA × DBA/2)F_1 Hybrid Mice of Experiments 2 and 3

In the (CBA × DBA/2)F_1 hybrid mice of experiments 2 and 3 the characteristics of the leukemias were identical in experimental and control mice. As apparent from tables 3 and 4, 96 cases of leukemia occurred among 213 mice. Leukemia consistently did not occur until the mice were very old, 18 months or over. The localization of gross and microscopic lesions corresponded to that usually seen in leukemia.

A definite classification into various types of leukemia is difficult because so many of these leukemias in old mice are rather atypical. Since electrophoretic studies showed that "takes" of one of the earliest plasma-cell leukemias to appear and be transplanted into F_1 mice were associated with the presence of an abnormal serum-protein fraction, as many as possible of the leukemias, which gradually appeared, were transplanted; the number of transplantation lines now surpasses fifty. The inoculated plasma-cell leukemias develop very slowly and their survival time in the first passage is often more than 12 months; in later passages the survival time gradually shortens.

These experiments are still in progress and will be published later. The final classification of the leukemias has been postponed until these transplantation lines are fully investigated, and probably will form a basis for a more well-founded classification of the leukemias from which they originated.

From present estimates, 20 of the 96 leukemias listed in tables 3 and 4 were of the lymphatic or stem-cell variety. Most of them in the original hosts, as well as in serial transfer, are characterized by a definitely leukemic blood picture, with as many as 900,000 white blood cells per mm^3.

About 50 leukemias are presumably plasma-cell leukemias of differentiation grades II to IV with transitions to reticulosarcomatosis, according to the criteria established previously (*17*, *18*). In the animals bearing transplants, the blood findings have usually included a relative granulocytosis, sometimes accompanied in later passages by a final outburst of plasma cells. At least 9 of these transplantation lines of leukemia are characterized by the occurrence of abnormal serum-protein fractions of the same type as that associated with human myelomas and plasma-cell leukemias.

A preliminary report on the first passages of two such leukemias has been published (*26–28*). One (transplantation line 20) was associated

with augmentation of the β-globulin fraction and the other (transplantation line 36) with the occurrence of a protein fraction between the β and γ ranges. Immunoelectrophoretic studies of serums from leukemic mice of both transplantation lines were recently performed (*29*) and, for comparison, serums from normal mice were investigated (*30*). These studies revealed that the paraprotein of line 20 is a γ-protein with β-mobility, that of line 36 a more slowly migrating γ-protein.

Five mice exhibited reticulosarcomatosis, which was generalized in 4 mice, and in 1, only the liver was affected. In later passages, reticulum cells were often observed in the blood.

Three mice had ovarian reticulosarcomas and 1 had a thymic reticulosarcoma with little involvement of the sites of predilection in leukemia. Subcutaneous transplantation of these 4 neoplasms resulted in only a small, local growth, whereas the spread was violent, particularly to the liver and spleen. In addition, there was severe jaundice.

One mouse had an ovarian tumor. Microscopic sections of this tumor suggested a granulosa-cell tumor, but imprint preparations showed numerous, rather monocyte-like cells, with peculiar azurophilic granules in the cytoplasm. No blood studies were made. Microscopic examination of the organs revealed no leukemic changes. Subcutaneous transplantation of the tumor gave rise to leukemia, presumably monocytic, with numerous cells in the blood like those mentioned, and with violent leukemic infiltration of the organs, especially the liver, whereas only very small growths developed at the site of implantation. The "takes" were accompanied by the development of an abnormal serum-protein fraction in the range between β- and γ-globulin.

A few leukemias could not be classified because of *post-mortem* changes. In several cases there was more than one histologic type of leukemic cell in the same mouse.

The development of leukemia observed shows that the (CBA × DBA/2)F_1 mice have a marked tendency to transform the reticular tissue into various types of leukemia.

In addition, it is worth mentioning that one mouse had a typical fibrosarcoma in the muscles of the right thigh, without metastasis. Transplantation through 17 passages has induced at the site of implantation subcutaneous tumors consisting of cells of the same spindle-shaped appearance and without metastasis. After the tumor tissue was frozen at −70° C. for 20 hours, the subcutaneous implant was converted into a typical reticulosarcoma, which showed, besides the local growth, infiltration of the liver, spleen, and lymph nodes. This reticulosarcoma has remained unchanged through 6 passages.

Discussion

Previous experiments (*14*) have shown that injection of 0.02 mg. of 7,12-dimethylbenz[*a*]anthracene, directly into the thymus *in situ* of DBA and C57BL mice, induced leukemia. The present experiments revealed that a corresponding carcinogenic action did not induce leukemia

in the implanted thymus of DBA and C57BL mice despite the fact that the implanted thymus was intact and regenerated at the time of injection. No explanation can be advanced.

Since injection of carcinogenic hydrocarbon directly into the thymus *in situ* in C3H mice did not lead to the development of leukemia (*14*), it is comprehensible that leukemia was not induced by carcinogenic action upon implanted C3H thymuses. The same applies to the absence of tumor development in the implanted thymuses from CBA mice, belonging to a typically low-leukemic strain, which, moreover, were not subjected to carcinogenic treatment.

It is less comprehensible that leukemic tumors did not develop in the implanted thymus from DBA/2 mice, a strain with a 30 to 40 percent incidence of leukemia (*19*). In corresponding experiments (*11*) with thymic tissue from strain RIL (in which, it is true, the incidence of leukemia is considerably higher), leukemic tumors were found in practically all the thymic grafts in leukemic mice.

The most remarkable finding in the present experiments was the acceleration of the host's development of leukemia, after implantation of the CBA thymus, *without* leukemic tumors being demonstrable in any of the grafts. In this respect, the present experiment differs from previous studies (*11*, *12*) in which the development of leukemia, even when it proved to consist of the host's cells, was invariably associated with the presence of leukemic tumors in the implanted thymus.

In connection with the observed acceleration of the development of leukemia, preferably plasma-cell leukemia, in mice with CBA thymic grafts, our earlier findings (*14*) may be taken into consideration. These showed that the incidence of plasma-cell leukemia was enhanced by direct injection of a small dose of carcinogenic hydrocarbon into the thymus *in situ*. Thus, this observation, too, indicates that the thymus may influence the development of leukemia of the plasma-cell type.

The development of leukemia, when a relatively large number of (CBA $\times$ DBA/2)F_1 mice were allowed to live until an advanced age, has been briefly described. Further details will be published later. In addition to a number of presumably lymphatic leukemias, a large proportion were plasma-cell leukemias, several showing abnormal serum-protein fractions, with even transition to pure reticulosarcomatoses and localized reticulosarcomas. The latter, however, proved to give infiltrating growth as reticulosarcomatoses when transplanted. This development of leukemia may be best characterized by stating that it corresponds exactly to the excellent and exhaustive description, by Dunn (*31*), of plasma-cell leukemia and reticulosarcomatosis in mice. The similarity is so close that all the rare manifestations collected by Dunn from mice of various strains occurred in our series of (CBA $\times$ DBA/2)F_1 mice. This must be interpreted as a manifestation of the marked capacity of these mice for leukemic transformation of the reticular tissue. Thus, this transformation demonstrates the close relationship between the various types of leukemia, including localized reticular neoplasms of different morphologic appearances.

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Studies on the Anemia of Tumor-Bearing Animals. II. The Mechanism of Erythrocyte Destruction [1,2]

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Summary

When Cr^{51}-labeled erythrocytes were transfused into rats bearing Lymphosarcoma R2788, the tagged erythrocytes disappeared more rapidly than in normal rats. Nearly all the radiochromium which disappeared from the blood stream was recovered in the tumor. Since radiochromate binds to globin in the red cell, the presence of noncirculating Cr^{51} in the tumor suggests that the protein moiety of hemoglobin, as well as the iron moiety, was deposited in the tumor. When Cr^{51}-tagged erythrocytes were lysed and then injected very slowly into tumor-bearing animals, the radiochromium disappeared very rapidly from the blood stream and was not deposited in the tumor but was recovered in the liver, spleen, kidneys, and urine. It is concluded that the erythrocyte iron did not enter the tumor by a process of intravascular hemolysis, followed with uptake of released hemoglobin by viable tumor cells, but entered the tumor in the intact red cell. These conclusions suggest that vascular injury may be a major factor in the pathogenesis of the erythrocyte destruction observed in the tumor-bearing animals.—J. Nat. Cancer Inst. 21: 1099–1107, 1958.

In the preceding paper (*2*) it was observed that transfused Fe^{59}-labeled erythrocytes disappeared from the circulation more rapidly in rats, bearing Lymphosarcoma R2788, Hepatoma 3683, Yoshida ascites tumor, Harderian gland carcinoma 2226, and Fibrosarcoma 4956, and in mice, bearing Fibrosarcoma HE8971, Hepatoma 134 (ascites), and spontaneous C3H mammary tumors, than in their corresponding normal controls. It was shown that a major portion of the erythrocyte iron which disappeared from the circulating blood could be recovered in the tumor, and that only small amounts were found in the liver and spleen. It appeared, from these experiments, that the large amount of iron reported previously in our rat

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tumors (*3*) was, for the most part, derived from erythrocyte iron. The degree of red cell destruction varied greatly with the tumor type and, when pronounced, it was accompanied by marked cachexia and early death of the host.

In several clinical reports dealing with the shortened survival of erythrocytes in patients with leukemia and solid tumors, it has been suggested that the accelerated destruction of erythrocytes was caused by a hemolytic process of an obscure nature (*4*, p. 102; *5*, *6*). These opinions were based primarily on the fact that a number of investigators had shown that tumor extracts have hemolytic properties (*7–14*), and that other investigators had observed evidence of erythrocyte injury in some patients and animals with cancer, indicated by the presence of spherocytosis, a positive Coombs' test, or an increased hypotonic fragility (*15–19*). Because of the prevailing opinion that red cell destruction in the cancer host resulted from the hemolysis of injured circulating erythrocytes, it was necessary to consider three possible mechanisms by which erythrocyte iron might be deposited in the rat tumors mentioned:

1) That as the tumor grew it released toxic substances or antibodies that damaged the erythrocytes and caused their hemolysis within the circulating blood, and that the released hemoglobin was utilized by viable tumor cells as a source of nitrogen. It has been suggested by several investigators that the proteins of the plasma may serve as an important source of tumor nitrogen (*20–23*).

2) That the substances from the tumor damaged the erythrocytes, which led to their phagocytosis in the spleen and reticuloendothelial system, and that, for some unknown reason, a large fraction of the released radioiron was taken up from the plasma by the tumor.

3) That the tumor destroyed its own blood supply, which caused hemorrhages and thromboses of sufficient magnitude to account for the red cell destruction, even in the absence of external hemorrhage.

The marked fluctuation in the rate at which Fe^{59}-labeled red cells left the blood stream in individual animals suggested that a hemorrhagic process was the more likely possibility. On the other hand, the apparent discrepancy between the amount of blood known to have escaped from the blood stream in a given period of time and the rather limited areas of hemorrhage grossly visible within the tumor appeared to belie a hemorrhagic process unless it were accompanied by a rapid process of erythrocyte destruction in the area of the tumor.

This paper presents experiments on the fate of hemoglobin of intact and lysed red cells in tumor-bearing animals. In these studies the protein moiety of hemoglobin was labeled with radiochromate to utilize an alternative labeling technique. The use of radiochromate offered an advantage because it does not enter into the many pathways of metabolism

normally involved in the utilization of iron. It was hoped that by tagging the red cell with Cr^{51} the radiochromium might be deposited at the site of red cell destruction.

The experiments show that when intact erythrocytes are injected into tumor-bearing animals, nearly all the radiochromium which has left the blood stream is recovered in the tumor. On the other hand, when the red cells are lysed and injected in very small amounts over a period of time, the radiochromium leaves the blood stream rapidly and does not go to the tumor but is recovered in the liver, spleen, kidneys, and urine. This indicates that the hemoglobin enters the tumor in intact red cells, and points to the importance of vascular lesions in the pathogenesis of the erythrocyte destruction occurring in these tumor-bearing animals.

Materials and Methods

The animals and tumors used and the techniques employed have, for the most part, been described in the preceding paper. In order to measure the amount of noncirculating radiochromium in a given tissue, the circulating blood was tagged with a second isotope by injection of Fe^{59}-labeled erythrocytes 30 minutes before the animal was killed. The amount of radiochromate in the circulating blood of each tissue, at the time the animal was killed, was calculated by the following formula, which is similar, but inverse, to that used in the preceding paper: Fe^{59} content of the organ $\times$ ratio of Cr^{51}/Fe^{59} in the circulating blood = amount of circulating Cr^{51} remaining in the organ. The amount of noncirculating radiochromium was calculated by subtracting the circulating radiochromium from the total radiochromium content of the tissue.

In certain studies lysed cells [6] were used instead of whole cells. In these experiments the blood was labeled by incubation, for 3 hours, with large quantities of radiochromate, as much as 100 μg. of chromium per ml. of blood, in order that the hemoglobin in 0.01 or 0.02 ml. of blood would contain sufficient radioactivity to allow tissue-distribution studies in rats. Then 0.25 ml. of packed, labeled erythrocytes were washed twice with 0.9 percent saline and lysed by the addition of 1 ml. of distilled water to the packed cells. After 20 minutes, the solution was diluted with 3.75 ml. of 1.8 percent NaCl and centrifuged at room temperature in an International centrifuge at 2000 rpm for 5 minutes. The supernatant was then assayed and diluted with saline to bring the hemoglobin to a concentration of about 2 mg. per ml. before injection.

Experiments

Studies with intact erythrocytes.—Chromate-labeled erythrocytes, containing 6 μg. of chromium with 4.3 μc of Cr^{51} in 0.5 ml., were injected into

[6] Although crystalline hemoglobin was used in preliminary experiments with comparable results, lysed cells were used directly in these experiments since the lysed cells more nearly duplicated the phenomenon of intravascular hemolysis. Avoidance of the crystallization procedure also minimized the possibility of denaturation of the hemoglobin and avoided the formation of small crystals frequently found in purified hemoglobin preparations. Isolation and repeated crystallization of chromate-labeled hemoglobin from the lysed cells showed that nearly all the radiochromium was bound to hemoglobin (*24, 25*).

the femoral vein of M520 × (A × C)F_1 male rats bearing Lymphosarcoma R2788 weighing about 15 percent of the body weight. After a 30-minute period of equilibration, a sample of tail blood was withdrawn to permit determination of the initial blood volume. Three days later, after the level of Cr^{51} in the tail blood had fallen to about 67 percent of the initial value, the rats were injected with 0.25 ml. of erythrocytes labeled with 0.34 μc of Fe^{59}. After the usual 30-minute period of equilibration, a tail sample was taken from which the dilution of Fe^{59} in the blood was calculated, to permit determination of the final blood volume. The animals were then killed, and the concentrations of Fe^{59} and Cr^{51} were determined in the various tissues. The noncirculating radiochromium was determined as described in the section on methods. The distribution of noncirculating radiochromium in the blood, tumor, liver, and spleen is shown in table 1. A high proportion of the radiochromium lost from the blood stream was recovered in the tumor, whereas the amount in the liver and spleen was very low. This experiment demonstrated that the chromium of labeled red cells was deposited in the tumor and gave presumptive evidence that the globin moiety of hemoglobin as well as the iron moiety had entered the tumor. Thus it was unlikely that damaged erythrocytes had been phagocytized by the spleen and the reticuloendothelial system, and that the iron released had been taken up by the tumor from the plasma. The experiment did not, however, distinguish between the other 2 possibilities: 1) that hemoglobin had entered the tumor in intact red cells which had been deposited in the sites of hemorrhage or thrombosis or 2) that hemoglobin had been utilized as a source of nitrogen by viable tumor cells after having been released into the plasma by a hemolytic process.

TABLE 1.—*Distribution of radiochromium 3 days after injection of intact erythrocytes into rats bearing Lymphosarcoma R2788**

Animal	Blood	Tumor	Liver	Spleen	Total
1	70. 7	35. 3	1. 1	2. 9	110. 0
2	71. 1	19. 7	1. 3	1. 1	93. 2
3	58. 5	23. 2	2. 3	0. 9	84. 9
Average	66. 8	26. 1	1. 6	1. 6	96. 1

*Presented in terms of percent of injected dose.

Studies with lysed erythrocytes.—In order to determine whether or not radiochromium had entered the tumor in the intact erythrocyte, the fate of lysed erythrocytes which had been tagged with radiochromate was studied in normal and in tumor-bearing rats. A suspension of lysed red cells in 0.25 ml. of saline, which contained 0.55 mg. of hemoglobin tagged with sodium chromate containing 0.3 μg. of chromium and 3.3 μc of Cr^{51}, was injected into a male M520 × (A × C)F_1 rat bearing Lymphosarcoma R2788 and into a normal control. The level of Cr^{51} in the circulating blood was determined on 15 μl. samples withdrawn from the tail. In both normal and tumor-bearing animals the amount of circulating Cr^{51}

fell rapidly to 30 percent of the initial level in 3 hours, and the remainder disappeared at a much slower rate. The similarity of the 2 curves suggested that there was no major difference in the fate of hemoglobin in the normal and the tumor-bearing animals. The excretion of chromium in the urine and feces was measured for 2 weeks and then the animals were killed and the concentration of radiochromium in the varous tissues was determined. Since the total circulating radiochromium at this time was less than 0.1 percent of the injected dose, no attempt was made to correct the tissues for the radiochromium of the circulating blood. The data obtained are presented in table 2. The liver, spleen, and kidneys had a high specific activity. Of the radiochromium injected, 35 to 40 percent was in the urine, about 15 percent was in the liver, and 15 percent in the gastrointestinal tract and feces. The amount in the feces may be unduly high (*cf. 26*) because of the difficulty in maintaining complete separation between the feces and urine. Only 2.3 percent was in the tumor, which weighed 40 gm. compared to the total body weight of 197 gm. After 2 weeks about half the radiochromium was still in the carcass; the rest had been excreted.

TABLE 2.—*Distribution of radiochromium 14 days after a single injection of lysed erythrocytes into a normal rat and a rat bearing Lymphosarcoma R2788*

Tissue	Radiochromium (*cts./min./gm.*)		Recovery (*percent*)	
	N*	T†	N	T
Tumor	—	260	—	2. 3
Liver	7, 180	8, 460	15. 8	14. 2
Spleen	27, 290	4, 070	4. 7	1. 4
Bone	1, 730	990	13. 0	4. 9
Lung	660	549	0. 2	0. 2
Kidneys	4, 510	18, 660	2. 2	7. 0
Muscle	321	181	10. 2	1. 7
Gastrointestinal tract	—	—	1. 2	0. 7
Other tissues	—	—	5. 5	3. 4
Urine	—	—	34. 6	40. 8
Feces	—	—	13. 0	14. 1
Total	—	—	100. 4	90. 7

*Normal animals.
†Tumor-bearing animals.

In the experiment with intact erythrocytes, the red cells had disappeared from the circulation over a 3-day period at an average rate of 1.75 mg. of hemoglobin per hour per 100 gm. of body weight. In this experiment lysed erythrocytes containing 1.4 mg. of hemoglobin per 100 gm. body weight were injected. The possibility existed that the rate of injection of lysed cells was too rapid and was not representative of a slower and more continuous release which might be expected if intravascular hemolysis were occurring within the animal. For this reason, a second experiment was carried out in which a tumor-bearing rat weighing 200 gm. was anesthetized and injected with lysed erythrocytes. A Klett

electrophoretic compensator [7] was used to prolong the injection over a period of hours; the maximum rate of injection was 0.2 mg. per hour per 100 gm. of body weight. On each of 3 successive days 0.6 mg. of hemoglobin containing 0.4 μg. of chromium with 0.3 μc of Cr^{51} was injected. Twenty hours after the last injection the animal was killed; the distribution of radiochromium in the various tissues is shown in table 3. It will be seen that the tumor, which weighed 15 percent of the body weight, contained only 4 percent of the radiochromium injected. The liver, spleen, kidneys, and urine were high in specific activity and, again, most of the isotope was in the liver and urine.

TABLE 3.—*Distribution of radiochromium after multiple slow injections of lysed erythrocytes into a rat bearing Lymphosarcoma R2788*

Tissue	Radiochromium (*cts./min./gm.*)	Recovery (*percent*)
Blood	2. 2	1. 5
Tumor	2. 1	3. 9
Liver	59. 2	28. 7
Spleen	45. 1	5. 8
Muscle	0. 8	4. 5
Kidneys	27. 1	2. 7
Urine	13. 1	36. 8
Other tissues	—	11. 7
Total	—	95. 6

A similar experiment was carried out with Hepatoma 3683 in which lysed erythrocytes containing 1.5 mg. of hemoglobin labeled with 0.7 μc of Cr^{51} containing 0.5 μg. of chromium were injected by means of a compensator, over a period of 3 hours. The animal was killed 2 hours later: 50 percent of the injected radiochromium was found in the liver; the spleen contained 7 percent, the kidneys and urine 6 percent, and the tumor 5 percent. It appears, therefore, that the labeled hemoglobin is cleared from the plasma primarily by the liver [8] where it is presumably degraded and the released chromium is excreted in the urine. At no time were more than small amounts of the isotope deposited in the tumor. This is in marked contrast to the fate of labeled hemoglobin injected in intact cells, in which a major fraction of the isotope lost from the blood stream was recovered in the tumor.

Discussion

These results show clearly that when intact red cells, in which the globin moiety of hemoglobin had been tagged with radiochromate, were transfused into animals bearing Lymphosarcoma R2788 and Hepatoma 3683, the isotope which disappeared from the blood stream was deposited in the tumor in a very high yield. In sharp contrast to this, when radiochromate-tagged red cells were lysed before their injection, the radio-

[7] Klett Manufacturing Co., 179 E. 87th St., New York, N. Y.

[8] These results are in general agreement with those obtained by Jandl *et al.* (27) after the injection of Cr^{51}-labeled hemoglobin into normal human subjects.

isotope was not deposited in the tumor but was found primarily in the liver and in the urine.

The fact that the radiochromium of tagged, intact erythrocytes as well as radioiron was deposited in the tumor (*2*) is presumptive evidence that erythrocyte iron was entering the tumor attached to the globin moiety. This renders unlikely the possibility that damaged erythrocytes were being removed from the circulating blood of the tumor-bearing animal by phagocytosis in the spleen or elsewhere in the reticuloendothelial system, and that released radioiron was being cleared from the plasma by the tumor. If defective circulating erythrocytes were being removed by phagocytosis in the spleen, it would also be expected that a large fraction of the radiochromium would have been deposited in that organ, as demonstrated by Jandl *et al.* (*27*, *29*).

The fact that the radiochromium of tagged, lysed erythrocytes was not deposited in the tumor, but was recovered in the liver and in the urine, essentially eliminates the possibility that red cells were being destroyed by intravascular hemolysis and that the released hemoglobin was being utilized by viable tumor cells as a source of nitrogen.

The fact that when red cells labeled with Fe^{59} (*2*) or Cr^{51} were transfused into tumor-bearing animals only a small fraction of the radioisotope was recovered in the liver and spleen, as compared to the amount deposited in the tumor, would seem to emphasize the importance of vascular injury in the pathway leading to erythrocyte destruction in these animals.

These studies emphasize the need for quantitative information on the fate of destroyed erythrocytes in the cancer patient in order to determine how much of the destruction is occurring as the result of direct injury to circulating erythrocytes as compared to injury from vascular damage in the area of the tumor. The problems and difficulties of such studies have been discussed elsewhere (*28*), but it would seem that our experiments and those of Jandl *et al.* (*27*, *29*) on the fate of defective erythrocytes could serve as a basis on which similar studies in the cancer patient could be developed. Recently Ultmann (*30*) followed the fate of Cr^{51}-labeled erythrocytes by a modification of the surface counting technique of Jandl (*27*), and showed that when labeled cells were transfused into some cancer patients, radioactivity was concentrated in the tumor masses, whereas in other patients high counting rates were obtained over the spleen. Such studies are a definite step forward in understanding the processes of erythrocyte destruction which occur in the cancer patient. Ultimately, however, greater quantitation than that offered by surface counting techniques will be required in order to evaluate how much red cell destruction is occurring by the various pathways in the cancer patient.

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Incorporation of Acetate-1-C^{14} Into Cholesterol by Human Intracranial Tumors *In Vitro* [1,2]

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Summary

Normal adult human brain does not incorporate C^{14}-labeled acetate into cholesterol *in vitro*. Under similar conditions, glioblastoma multiforme, astrocytomas, meningiomas, neurofibromas, acoustic neurinomas, and ependymomas can accomplish this conversion. The synthetic pathway in the tumors appears to be similar to that in normal liver tissue. Cholesterol biosynthesis in human central nervous system tumors is inhibited by oral administration of vanadium as diammonium oxytartratovanadate.—J. Nat. Cancer Inst. 21: 1109–1115, 1958.

In an attempt to find an accessible human tissue for the study of factors influencing endogenous cholesterol synthesis, the brain tumor was considered, since this tissue is obtained by surgical excision on the same patient on two occasions. Although it has recently been reported that normal adult rat brain can convert acetate-C^{14} into cholesterol, when injected either intracerebrally (*1*) or intracisternally (*2*), surviving tissue slices of adult rat (*3*) and rabbit brain (*4*) cannot accomplish this conversion. However, slices of the brain of very young animals do convert acetate to cholesterol (*3*). Brante (*5*) states that the lipide composition of brain tumors is strongly reminiscent of that of the brain in the early fetal stages. Assuming that neoplastic brain tissue is a reversion to a more primitive type, with a greater permeability, we studied the incorporation of acetate-1-C^{14} into cholesterol by normal and neoplastic human brain tissue. All brain tumors studied to date have shown incorporation of acetate into cholesterol. Since human brain tumors do synthesize cholesterol, it was possible to demonstrate that vanadium, a potent inhibitor of cholesterol biosynthesis in animals (*6*), also produces a similar inhibition in human tissue.

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[5] This work was performed during tenure of an Established Investigatorship of the American Heart Association.

Materials and Methods

Viable brain tissue was obtained at craniotomy by sharp dissection, before its blood supply was severed, and immediately placed in iced phosphate buffer (*7*). Slices were prepared with a Stadie-Riggs microtome. Every effort was made to utilize only pure tumor, but histologic control over each slice was impossible. From 1 to 3 gm. of tissue was incubated in phosphate buffer with 10 mg. sodium acetate (0.5 mc./mmole) or 2 mg. mevalonic acid-2-C^{14} (0.82 mc./mmole) for 2 hours at 37° C., in an atmosphere of 95 percent oxygen and 5 percent carbon dioxide, and the mixture was saponified in ethanolic KOH for 1 hour under reflux. The period from the excision of the tissue to the start of incubation never exceeded 20 minutes. The nonsaponifiable fraction was extracted with petroleum ether (b.p. 30 to 60° C.) and the cholesterol isolated as the digitonide (*8*). The cholesterol was purified as the dibromide (*9*) and its radioactivity determined with an end-window Geiger-Mueller tube. Enough counts were obtained so that 2 counts above background were reliably higher than ambient ionization counts at a level of confidence less than 0.01. A small piece of tissue was placed in fixative for morphologic evaluation.

An aliquot of the slices prepared for incubation was dried over phosphorus pentoxide in a vacuum oven at 56° C. The dry tissue was extracted for 1 hour with acetone-alcohol (1:1) at 80° C., and then overnight at 37° C. The total and free cholesterol content of the tissues was determined by the method of Sperry and Webb (*10*).

The patients given vanadium received diammonium oxytartratovanadate [6] orally for a variable period before craniotomy. The ability of the tumors to incorporate acetate-C^{14} into cholesterol was determined, and the tissue removed from the patients receiving vanadium was analyzed for vanadium content (*11*).

Results

Normal human brain does not synthesize cholesterol [7] *in vitro* whereas normal intestinal mucosa does (table 1). Under similar conditions all brain tumors studied to date convert acetate to cholesterol. The cholesterol concentration for white matter of the normal brain was 124 to 143 mg. per gm. dry tissue (table 1), which agrees with Brante (*5*), who also found that gray matter had a concentration of approximately 5 percent total cholesterol. This finding explains the lower cholesterol concentration in tissue samples containing both white and gray matter.

The different tumors show no correlation between morphologic type, rate of synthesis, and total or ester cholesterol (table 2). However, as a group, the tumors show a decrease in cholesterol concentration from that of normal brain tissue and an increase in the amount of esterified cholesterol. The activity of metastatic tumors will, no doubt, depend upon

[6] Supplied by Merck Sharp & Dohme Division of Merck & Co., Inc., Rahway, N. J.

[7] Synthesis is defined as incorporation of C^{14}-labeled acetate into cholesterol purified via dibromide formation.

TABLE 1.—*Incorporation of acetate-1-C^{14} into cholesterol by normal human brain*

Normal brain*	Cholesterol		Radioactivity†
	Mg./gm. dry tissue	Percent ester	
Occipital (W)	143	1	0
Frontal (W)	124	3	0
Frontal (W)	—	—	0
Parietal (W and G)	62. 7	3	0
Occipital (W and G)	78. 8	5	0
Frontal (W and G)	82. 0	—	0
Cerebellum (W and G)	63. 3	3	0
Normal sigmoid mucosa	12. 4	—	18, 340

*W=white matter. G=gray matter.
†Radioactivity=cpm per mg. cholesterol × mg. cholesterol per gm. dry tissue.

the type of the primary tumor. The slight variability and somewhat higher values of our tumor cholesterol concentrations in comparison to those reported by Brante (*5*) and Randall (*12*) can be explained by the heterogeneous composition of some of our tumor samples.

In contrast to the intracranial tumors, a human adenocarcinoma of the sigmoid colon (table 2) incorporated labeled acetate into cholesterol at a much slower rate than normal mucosa, although the concentration of cholesterol was greater in the tumor.

There was no cholesterol synthesis (table 3) in brain tumors removed from patients after vanadium was administered, even though synthesis

TABLE 2.—*Incorporation of acetate-1-C^{14} into cholesterol by human intracranial tumors*

Tumors	Cholesterol		Radioactivity*
	Mg./gm. dry tissue	Percent ester	
Astrocytoma	29. 3	—	603
	16. 6	21	209
Glioblastoma multiforme	93. 4	65	5, 090
	26. 6	69	4, 250
Metastatic tumors	19. 4	71	3, 410
	39. 0	45	964
Meningioma	13. 3	49	10, 140
	23. 0	—	1, 400
	12. 0	12	857
Neurofibroma	16. 7	6	2, 878
Acoustic neurinoma	31. 1	24	1, 870
Ependymoma	54. 6	15	22, 600
Sigmoid mucosa			
Normal	12. 4	—	18, 340
Carcinoma	39. 2	—	4, 050

*Radioactivity=cpm per mg. cholesterol × mg. cholesterol per gm. dry tissue.

TABLE 3.—*Effect of vanadium administration upon incorporation of acetate-1-C^{14} into cholesterol by human brain tumors*

Patient	Age	Administration of 100 mg. diammonium oxytartratovanadate (*days*)	Craniotomy	Radioactivity* tumor cholesterol	Tumor vanadium concentration (*μg./gm. dry tissue*)	Morphologic diagnosis
G. E.	43	15	2nd	0	1. 2	Astrocytoma
J. W.	47	0	2nd	1030	—	Glioblastoma multiforme
B. A.	57 58	0 30	1st 2nd	684 0	— 1. 5	Glioblastoma multiforme

*Radioactivity = cpm per mg. cholesterol × mg. cholesterol per gm. dry tissue.

of cholesterol was present in the tumor of one of the patients previous to vanadium administration. The vanadium concentration in the tumor of G.E. was 1.2 μg. per gm. dry tissue, and 1.5 μg. per gm. dry tissue in the tumor of B.A. These concentrations are greater than those previously shown necessary to produce 70 percent inhibition of rabbit liver cholesterol biosynthesis (*13*).

Discussion

These data show that all types of human intracranial tumors studied synthesize cholesterol *in vitro* whereas normal adult human brain does not. The normal brain is not devoid of synthetic ability since it was able to convert the acetate to a radioactive digitonin-precipitable substance that was not cholesterol because all radioactivity disappeared with the preparation of the dibromide. Synthesis of such a digitonin-precipitable substance has also been demonstrated in Yoshida ascites cell tumors (*14*) and in the aortas of some carnivorous and omnivorous animals (*8*).

Unlike tumors of other tissues, brain tumors synthesize cholesterol more rapidly than the tissue from which they arise. For example, Emmelot and Bosch (*15*) demonstrated that tumors such as rat hepatomas do not synthesize cholesterol as rapidly as the corresponding normal tissue. To compare intracranial tumors with other human tumors, an adenocarcinoma of the sigmoid colon was studied (table 2); this tumor incorporated labeled acetate into cholesterol at a much slower rate than the adjacent normal mucosa. Calculations by Medes, Thomas, and Weinhouse (*16*) indicate that hepatomas do not synthesize fatty acids rapidly enough to account for their concentration within the tumor, and that the tumor must draw on the supplies of the host for a good share of its requirements. The same also appears to be true of the cholesterol requirements of the carcinoma of the sigmoid colon since the cholesterol concentration is 3 times that of normal mucosa, while the rate of synthesis of cholesterol is only one-fourth normal. Indeed, Schwenk and Stevens (*14*) have recently demonstrated concentration of exogenous cholesterol-C^{14} by Yoshida ascites cell tumor.

Tumors of the patients who received vanadium did not synthesize cholesterol (table 3). Since all other intracranial tumors studied synthesize cholesterol, since the tumor removed from B.A. synthesized cholesterol before he received vanadium, and since enough vanadium was present in the tumor to inhibit synthesis, it must be concluded that vanadium inhibits cholesterol synthesis in human brain tumors. It could not be shown whether or not the tumor of G.E. synthesized cholesterol before he received vanadium because his first craniotomy was performed several years before this study was undertaken. To determine whether or not the results of a previous craniotomy *per se* could be the inhibiting factor of a second operation, a tumor of a third patient, J.W., was studied during a second craniotomy. This man did not receive vanadium and his tumor retained its synthetic ability, even though he had received 2 courses of deep X-ray therapy prior to his second operation.

The following compounds have been suggested as intermediates in cholesterol biosynthesis: acetoacetate, β-hydroxy-β-methylglutaric acid,[8] β-methylcrotonic acid,[9] mevalonic acid, squalene, and lanosterol. To determine whether intracranial tumors synthesize cholesterol along the same metabolic pathways as those used by liver tissue, in which the above intermediates were originally detected, we isolated (*17*) β-hydroxy-β-methylglutaric acid, β-methylcrotonic acid, and squalene from a meningioma incubated with C^{14}-labeled acetate and found radioactivity in each compound. This activity was retained by the derivative prepared from each compound. Intracranial tumors were also incubated with mevalonic acid-2-C^{14}, and radiocholesterol was isolated after the incubation. The presence of radioactivity in all the compounds isolated plus the ability of the tumors to convert mevalonic acid to cholesterol strongly suggests that the synthetic pathway is the same in brain tumors as in normal liver.

One requirement of the ideal inhibitor of cholesterol biosynthesis is that the compound be shown to inhibit human tissue cholesterol synthesis (*18*). The fulfillment of this criterion for vanadium is satisfied here with the demonstration of inhibition of cholesterol synthesis in human intracranial tumors. In addition, the demonstration of inhibition of human tissue cholesterol synthesis suggests the use of such inhibitors as a form of therapy for brain tumors in man. The efficacy of such compounds would depend upon whether cholesterol were a metabolic requirement of the tumor rather than merely a structural component. Even if cholesterol does prove to be a metabolic requirement of the tumor, inhibitors may not prove effective in slowing tumor growth because of the possibility of the tumor obtaining the necessary cholesterol from the circulating plasma pool. Studies to evaluate vanadium as an agent for the therapy of brain tumors are now in progress. However, preliminary results indicate this type of inhibitor is not an effective antitumor compound.

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Treatment of Mouse Lymphosarcoma by Total-Body X Irradiation and by Injection of Bone Marrow and Lymph-Node Cells [1,2]

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Summary

The lymphosarcoma could not be eliminated by total-body X irradiation with a supralethal dose. This is in accordance with the experience of other workers who treated transplantable tumors in animals by total-body irradiation. Injection of homologous or heterologous lymph-node cells in addition to bone marrow resulted in inhibition of tumor growth. However, the animals ultimately died either of lymphosarcoma or foreign bone-marrow reaction. It has been shown that antibodies produced by homologous or heterologous lymph-node cells probably play a major part in the inhibition of tumor growth. On the other hand, the debilitating effect of the foreign bone-marrow reaction cannot be excluded with certainty as a factor in the inhibition of tumor growth. The best results with respect to the survival of the animals were obtained after the injection of large numbers of isologous lymph-node cells. In the latter case, the inhibition of lymphosarcoma growth probably is a consequence of competitive proliferation of normal and irradiated neoplastic lymphoid cells in the irradiated host. In these experiments competitive proliferation could not be demonstrated after injection of large numbers of homologous lymph-node cells since this procedure is acutely lethal to mice.—
J. Nat. Cancer Inst. 21: 1117–1129, 1958.

In the treatment of tumors of the hematopoietic system, total-body X irradiation seems to be preferable to local irradiation because of the extensive dissemination of the malignancy. With the aid of bone-marrow transplantation the impending bone-marrow syndrome can be prevented, and high doses of total-body irradiation may be administered in an attempt to eliminate the tumor in experimental animals (*1–8*). The X-ray dose that may be applied is limited by the risk of intestinal radiation death which occurs in mice after doses above 1000 r. The intestinal radiation death cannot be prevented by bone-marrow transplantation. Therefore the success of the therapy by total-body irradiation will be dependent on the radiosensitivity of the tumor cells.

[1] Received for publication July 18, 1958.
[2] A preliminary report on part of the investigation was presented at the Seventh International Cancer Congress.

The varying results obtained in animals when tumors of the hematopoietic system are treated by total-body irradiation and injection of hematopoietic cells are probably a reflection of differences in the radiosensitivity of the tumors.

Barnes *et al.* (*2*) suggested that tumor cells, which survive the irradiation, could be eliminated by antibodies produced by a graft of homologous hematopoietic tissue. An immunological reaction of the graft against the host probably occurs in mice that have been protected against acute radiation death by transplantation of homologous bone marrow and may be one of the causes of secondary death of the animals (*8*). It might be expected that the antibodies which are directed against the tissues of the host will also be noxious toward tumor cells of the host's genotype.

We have studied the effect of a lethal dose of total-body irradiation on the growth of a transplantable lymphosarcoma of the C57BL mouse. The mice inoculated with the tumor were treated after irradiation with isologous, homologous, or heterologous (rat) bone marrow. Since lymphoid cells are known to be important in the rejection of homografts, groups of animals were treated with isologous, homologous, or heterologous lymph-node cells in addition to bone marrow.

Materials and Methods

The lymphosarcoma.—The lymphosarcoma originated in an unirradiated C57BL female. The tumor had been maintained in C57BL females by intravenous injection of spleen suspensions. After inoculation in C57BL mice the tumor seldom affects the thymus. Otherwise the tumor behaves as a typical mouse lymphoma. Thus far the lymphosarcoma has been found to be aleukemic. Microscopically the tumor cells were of the immature lymphoblastic type.

Mice.—The irradiated mice were C57BL males and (CBA ♂ × C57BL ♀)F_1 males, 7 to 12 weeks of age, weighing 20 to 28 gm. and 20 to 30 gm., respectively. The F_1 hybrids were obtained by breeding C57BL females with CBA males. Both the C57BL and the CBA strains were obtained in 1953 from Professor Dr. O. Mühlbock, Antoni van Leeuwenhoekhuis, Amsterdam, and have since been maintained by strict brother × sister inbreeding.

C57BL, CBA, and (CBA ♂ × C57BL ♀)F_1 females, 6 to 8 weeks of age, were used as donors of the bone marrow and of lymph-node cells. Young female rats of an inbred Wistar albino strain (WAG), 6 to 8 weeks of age and weighing 100 to 120 gm., were used as donors of rat bone marrow and lymph-node cells.

The suspensions of lymphosarcoma cells were prepared from spleens of female C57BL mice inoculated with the tumor at least 6 days before.

All animals had access *ad libitum* to a commercial cubed food. Details on the pre- and postirradiation sanitation of the animals have been presented elsewhere (*9*).

Irradiation.—The mice were irradiated in groups of 10 or 15 in a circular perspex cage. The physical constants of the X radiation were:

200 kv., 20 ma., filtration, 1.5 mm. of Cu, HVL, 1.8 mm. of Cu. The target distance was 50 cm. and the dose rate was 45 r per minute. The X-ray dose was 800 r in all experiments. The dosage was measured on the bottom of the perspex box with a Baldwin substandard ionization chamber. Maximal backscatter was used. The relation between X-ray dose and mortality of the mouse strains employed has been presented elsewhere (*10*). The LD100/30 dose is about 700 r for our strains.

Cell suspensions.—Suspensions of hematopoietic tissues and lymphosarcomatous spleens were prepared by squeezing the mashed tissue through nylon-gauze filters. Suspensions of lymph nodes were freed of fat droplets by centrifugation. Bone marrow was obtained from the femurs of mice and rats. A hemacytometer was used in estimating the number of nucleated living cells in Tyrode's solution containing 0.2 percent eosin. The cells that stained with eosin were considered to be dead.

The cell suspensions were injected into the tail vein in a volume of 0.5 ml. within 4 hours after the irradiation, except in one experiment, which will be described separately.

Bone marrow and lymph-node cells were suspended in Tyrode's solution; lymphosarcoma cells in a 0.9 percent NaCl solution which contained 10,000 I. U. of sodium penicillin per ml.

Results and Discussion

Experiment 1

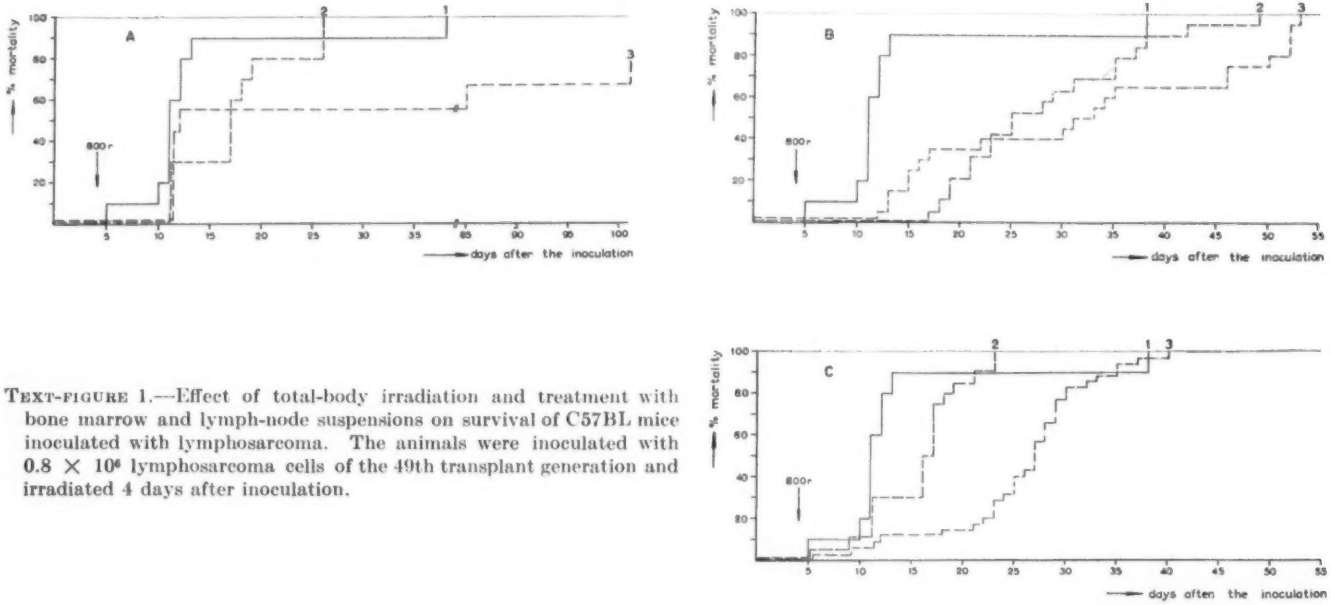
Male C57BL mice, 7 to 12 weeks of age, were inoculated with 0.8 $\times$ 10^6 lymphosarcoma cells and irradiated with 800 r 4 days after the inoculation. The animals were divided into 2 groups.

The animals of the first group were treated with isologous, homologous (CBA), or heterologous (rat) bone marrow. The animals of the second group received isologous, homologous, or heterologous bone marrow and lymph-node suspensions. The isologous and homologous cell suspensions and the heterologous bone marrow were administered within 4 hours after the irradiation. The heterologous lymph-node suspension was injected 24 hours after the irradiation.

A number of animals treated with isologous or heterologous cell suspensions died between the 5th and the 12th day after the inoculation (text-fig. 1, A and C). *Post-mortem* examination showed normal or small spleens in all these animals (text-fig. 2, A and C). In contrast, the unirradiated, untreated, tumor-bearing control mice, which died in the same period, had large spleens showing infiltration with tumor tissue.

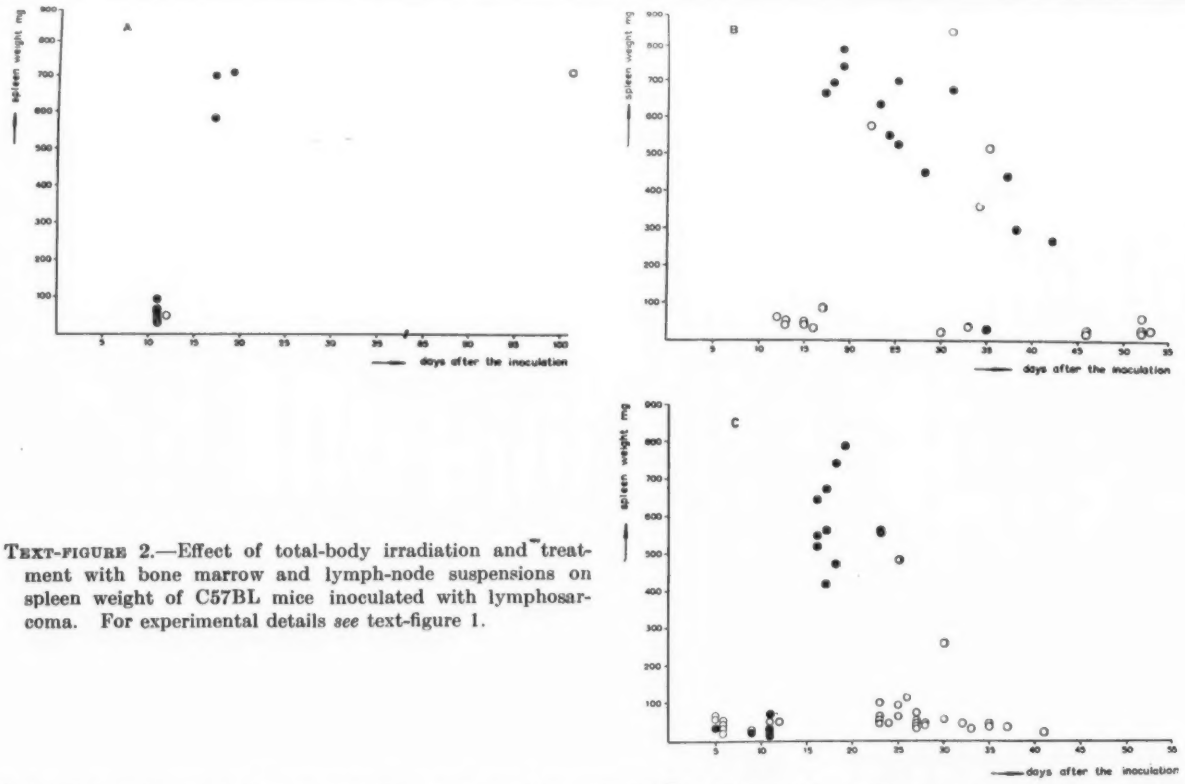
The majority of the animals treated with isologous bone marrow lived somewhat longer than the unirradiated, untreated controls (text-fig. 1, A). The mice that died after the 12th day following the inoculation showed enlarged spleens and other signs of lymphosarcoma (text-fig. 2, A).

After treatment with isologous bone marrow and lymph-node suspensions some of the mice survived considerably longer (text-fig. 1, A). Two animals ultimately died of lymphosarcoma on the 85th and the 101st day, respectively, and 2 were still alive after 112 days.



TEXT-FIGURE 1.—Effect of total-body irradiation and treatment with bone marrow and lymph-node suspensions on survival of C57BL mice inoculated with lymphosarcoma. The animals were inoculated with 0.8×10^6 lymphosarcoma cells of the 49th transplant generation and irradiated 4 days after inoculation.

A = Isologous bone marrow and lymph node: 1) unirradiated, untreated controls, 10 mice; 2) 800 r and bone marrow, 1×10^6 cells, 10 mice; 3) same as 2, plus lymph-node suspension, 14×10^6 cells, 9 mice. **B** = Homologous bone marrow and lymph node: 1) unirradiated, untreated controls—same 10 mice as in A1, above; 2) 800 r and bone marrow, 13×10^6 cells, 19 mice; 3) same as 2, plus lymph-node suspension, 0.1×10^6 cells, 20 mice. **C** = Heterologous bone marrow and lymph node: 1) unirradiated, untreated controls—same 10 mice as in A1, above; 2) 800 r and bone marrow, 36×10^6 cells, 20 mice; 3) same as 2, plus lymph-node suspension, 20×10^6 cells, 35 mice.



TEXT-FIGURE 2.—Effect of total-body irradiation and treatment with bone marrow and lymph-node suspensions on spleen weight of C57BL mice inoculated with lymphosarcoma. For experimental details *see* text-figure 1.

A = Isologous bone marrow and lymph node: ● = isologous bone marrow; ○ = isologous bone marrow and lymph-node suspension. **B** = Homologous bone marrow and lymph node: ● = homologous bone marrow; ○ = homologous bone marrow and lymph-node suspension. **C** = Heterologous bone marrow and lymph node: ● = heterologous bone marrow; ○ = heterologous bone marrow and lymph-node suspension.

Treatment with homologous bone marrow resulted in a prolonged survival as compared to the controls (text-fig. 1, B). All except 1 of the animals died of lymphosarcoma (text-fig. 2, B). As these animals also lived longer than the mice treated with isologous bone marrow, other factors in addition to the irradiation must have influenced the growth of the tumor.

The existence of such factors was even more apparent after treatment with homologous bone marrow and lymph-node suspensions. Although a survival pattern was obtained similar to that after treatment with bone marrow only (text-fig. 1, B), the majority of the mice given homologous lymph-node suspension showed no signs of lymphosarcoma at autopsy. The spleens of 15 of the 19 animals were of normal size or even smaller (text-fig. 2, B).

A similar effect was found after injection of heterologous cell suspensions. The survival pattern of the mice treated with heterologous bone marrow only, did not differ appreciably from that of the mice treated with isologous bone marrow (text-fig. 1, C). The animals that died after the 12th day showed growth of the lymphosarcoma (text-fig. 2, C).

The mice treated with heterologous bone marrow and lymph-node suspension survived significantly longer than the mice treated with bone marrow only (text-fig. 1, C). Although all mice died, the majority showed no gross evidence of lymphosarcoma. Of 28 animals that died after the 12th day, 26 had a normal or small spleen (text-fig. 2, C).

Discussion.—The majority of the unirradiated, untreated control mice died of lymphosarcoma between the 5th and the 12th day after the inoculation, with extremely enlarged spleens. A number of the mice treated with isologous or heterologous cell suspensions died in the same period, but these showed normal or small spleens. Possibly the early death of the irradiated animals is a consequence of massive destruction of lymphosarcoma cells by the irradiation.

The mice treated with isologous bone marrow survived only slightly longer than the controls and all died of lymphosarcoma. This shows that the tumor is relatively resistant to a dose of 800 r.

The favorable effect of the treatment with isologous lymph-node cells on the survival of the mice will be discussed in connection with the results of the third experiment.

Treatment with homologous bone marrow and with homologous or heterologous bone marrow and lymph-node suspensions resulted in prolonged survival of a large number of mice. After treatment with homologous or heterologous bone marrow and lymph-node suspensions, the growth of the lymphosarcoma was definitely inhibited as was revealed by the finding of normal or even small spleens.

The results may be explained by presuming that the homologous and heterologous lymphoid cells have reacted immunologically against antigenic material of the C57BL mouse, with an inhibition of the growth of the lymphosarcoma. Since the animals had no gross signs of lympho-

sarcoma when they died, it must be inferred that the antibodies [3] have been active against the host as well. This explanation is supported by the fact that the injection of homologous lymphoid cells is also lethal for irradiated normal mice (*11*, *12*). Unfortunately, by varying the dose of homologous lymphoid cells, it has not been possible to obtain destruction of the tumor without killing the host.

Barnes and Loutit (*13*) have recently reported results which are in agreement with our findings. They point out that the wasting of the animals caused by the foreign bone-marrow reaction may be another factor in the inhibition of tumor growth.

Experiment 2

In this experiment the effect obtained after injection of homologous cell suspensions was further investigated.

Hybrid (CBA ♂ × C57BL ♀)F_1 males were inoculated with 20×10^6 cells of the C57BL lymphosarcoma maintained in C57BL females. This rather large amount of lymphosarcoma cells was found necessary to produce a 100 percent mortality in the F_1 hybrids.[4]

The mice were irradiated with 800 r and divided into 6 groups. After the irradiation, 3 groups were treated with isologous, C57BL, and CBA bone-marrow cells, respectively. The other 3 groups were treated with corresponding amounts of bone marrow and received, in addition, isologous, C57BL, and CBA lymph-node cells, respectively.

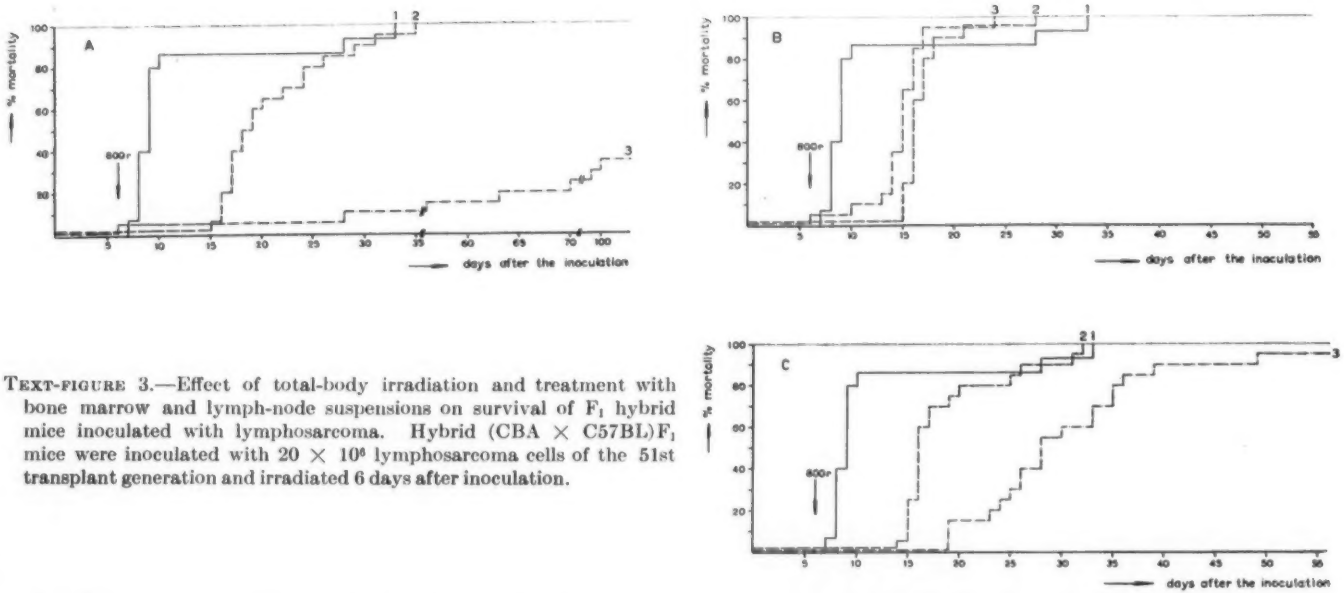
The survival pattern of the mice treated with isologous, C57BL, or CBA bone marrow was nearly identical (text-fig. 3: A, B, and C). All the animals died of lymphosarcoma. The prolongation of survival of mice treated with bone marrow only, is clearly to be attributed to the irradiation because irradiated, untreated, tumor-bearing mice died at the same time.

The majority of mice treated with isologous bone marrow and lymph-node suspension lived much longer than the mice of all other groups (text-fig. 3, A). The survival was even better than in experiment 1, since 13 of the 20 (65%) mice treated with isologous lymph-node suspension were still alive on the 100th day after inoculation. The other 7 mice died of lymphosarcoma.

A notable difference in survival was found between the mice treated with C57BL and those treated with CBA lymph-node suspension. The animals treated with C57BL bone marrow and lymph-node suspension died of lymphosarcoma simultaneously with the mice given bone marrow only (text-fig. 3, B). In contrast, treatment with CBA bone marrow and lymph-node suspension caused a prolongation of the survival compared

[3] In this paper we have used the term "antibodies" to designate an immunological activity similar to that which operates in the production of transplantation immunity. Admittedly humoral antibodies as well as cellular factors may be involved.

[4] The relative resistance of the F_1 mice toward the C57BL lymphosarcoma was rather unexpected. Skin transplants of our C57BL females have always taken in our F_1 hybrid males, and we have at present no explanation for the decreased transplantability of the lymphosarcoma in our F_1 hybrids.



TEXT-FIGURE 3.—Effect of total-body irradiation and treatment with bone marrow and lymph-node suspensions on survival of F_1 hybrid mice inoculated with lymphosarcoma. Hybrid (CBA × C57BL)F_1 mice were inoculated with 20×10^6 lymphosarcoma cells of the 51st transplant generation and irradiated 6 days after inoculation.

A = Isologous bone marrow and lymph node: 1) unirradiated, untreated controls, 10 mice; 2) 800 r and bone marrow, 1×10^6 cells, 20 mice; 3) same as 2, plus lymph-node suspension, 20×10^6 cells, 20 mice. **B** = C57BL bone marrow and lymph node: 1) unirradiated, untreated controls—same 10 mice as in A3, above; 2) 800 r and bone marrow, 1×10^6 cells, 20 mice; 3) same as 2, plus lymph-node suspension, 0.1×10^6 cells, 20 mice. **C** = CBA bone marrow and lymph node: 1) unirradiated, untreated controls—same 10 mice as in A3, above; 2) 800 r and bone marrow, 0.7 to 1×10^6 cells, 20 mice; 3) same as 2, plus lymph-node suspension, 0.1×10^6 cells, 20 mice.

to the treatment with CBA or C57BL bone marrow only (text-fig. 3, C), although all mice died ultimately of lymphosarcoma.

Discussion.—In this experiment a C57BL tumor was inoculated in a (CBA ♂ × C57BL ♀)F_1 host. An immunological reaction of the host against the lymphosarcoma is not to be expected. Hematopoietic cells derived from C57BL mice may be immunologically active against the F_1 host but not against the tumor. On the other hand, a graft of CBA origin may be expected to react against both the F_1 host and the C57BL tumor.

The outcome of this experiment is in complete agreement with the explanation given in the preceding section, namely, that the effect of homologous lymphoid cells on the growth of the lymphosarcoma is, at least partly, due to antibody production against the tumor. In this particular experiment there is little evidence that the inhibition of tumor growth following the treatment with homologous cells is secondary to the debilitating effect of the foreign bone-marrow reaction (*13*). Presumably both CBA and C57BL lymphoid cells may produce antibodies against the F_1 host. However, only the mice treated with CBA lymph-node cells lived longer than the mice treated with bone marrow only.

All the animals treated with C57BL and all but one of the animals treated with CBA bone marrow and lymph-node suspensions died of lymphosarcoma. In this respect the results of experiment 2 differ from those of experiment 1. In experiment 1 the majority of the animals treated with homologous lymph-node cells probably died due to the foreign bone-marrow reaction without showing any evidence of lymphosarcoma. It is typical of our F_1 hybrid, however, that very little secondary mortality occurs after treatment with parental bone marrow (*10*). It seems likely that the F_1 animals treated with CBA lymph-node cells lived long enough for the tumor to overcome the inhibiting effect of the immunological reaction.

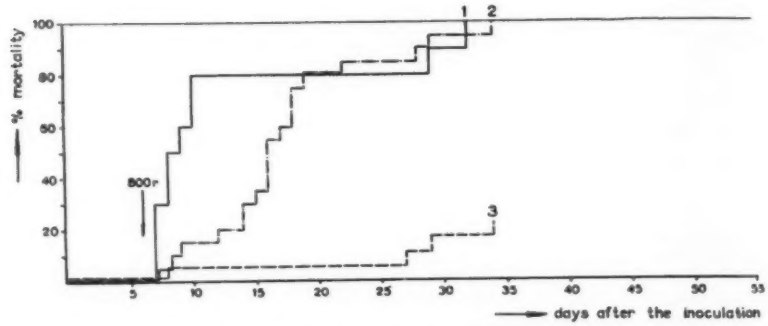
It is similarly conceivable that in the C57BL mice of experiment 1, which died after treatment with homologous or heterologous lymph-node cells without gross signs of lymphosarcoma, the tumor was not completely eliminated. The other possibility is that, in the latter case, recurrence of the tumor was prevented by the wasting of the animals caused by the foreign bone-marrow reaction.

Experiment 3

In this experiment the effect of isologous lymph-node cells on the growth of the lymphosarcoma was further investigated.

Hybrid (CBA ♂ × C57BL ♀)F_1 males were inoculated with 20×10^6 lymphosarcoma cells. Six days after the inoculation 2 groups were irradiated with 800 r. One group of 20 mice was treated with 1×10^6 isologous bone-marrow cells and 0.2×10^6 isologous lymph-node cells. The other group, consisting of 18 animals, received 1×10^6 isologous bone-marrow cells and 17×10^6 isologous lymph-node cells.

The survival pattern of the mice is given in text-figure 4. Comparison with text-figure 3A shows that the survival of the mice treated with



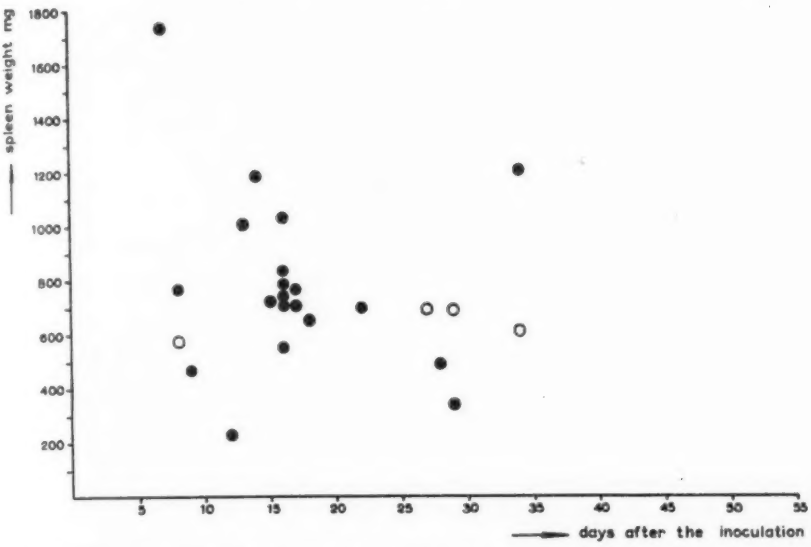
TEXT-FIGURE 4.—Effect of total-body irradiation and treatment with isologous bone marrow and lymph-node suspensions on the survival of F_1 hybrid mice inoculated with lymphosarcoma. Hybrid (CBA × C57BL)F_1 mice were inoculated with 20×10^6 lymphosarcoma cells of the 58th transplant generation and irradiated 6 days after the inoculation.

1) Unirradiated, untreated controls, 10 mice; 2) 800 r, 1×10^6 bone-marrow cells, and 0.2×10^6 lymph-node cells, 20 mice; 3) 800 r, 1×10^6 bone-marrow cells, and 17×10^6 lymph-node cells, 18 mice.

isologous bone marrow and 0.2×10^6 isologous lymph-node cells and those treated with isologous bone marrow, only, is almost similar. The survival of the mice treated with 17×10^6 isologous lymph-node cells was much more prolonged. Although the experiment is not yet completed, it appears that quantitatively the same effect was obtained as in experiment 2 after injection of 20×10^6 lymph-node cells. No differences in spleen weight were found between the animals treated with 0.2×10^6 and the 4 animals that died after treatment with 17×10^6 isologous lymph-node cells (text-fig. 5).

Discussion.—Various explanations may be given for the inhibition of growth of the lymphosarcoma after treatment with isologous lymphoid cells. The possibility exists that the lymphosarcoma has antigenic properties with respect to the host. In that case antibodies might have been formed against the tumor by the isologous (C57BL or F_1 hybrid) lymphoid cells. In experiment 2, the F_1 hybrids inoculated with the lymphosarcoma and treated after the irradiation with CBA bone marrow and 0.1×10^6 CBA lymph-node cells survived significantly longer than the mice treated with F_1, C57BL, or CBA bone marrow only, which indicates that this number of lymph-node cells is sufficient to evoke an immunological reaction against the tumor. In contrast, after treatment of the F_1 mice with 0.2×10^6 isologous or with 0.1×10^6 C57BL lymph-node cells, the survival time was not increased as compared to the treatment with bone marrow only. This suggests that immunological factors are not likely to be involved in the beneficial effect of the larger amounts of isologous lymph-node cells.

It seems probable that the growth-inhibiting effect on the tumor is dependent on the presence of large quantities of isologous lymphoid cells



TEXT-FIGURE 5.—Effect of total-body irradiation and treatment with isologous bone marrow and lymph-node suspensions on the spleen weight of F_1 hybrid mice inoculated with lymphosarcoma. For experimental details *see* text-figure 4.

● = 800 r, bone marrow, and 0.2×10^6 lymph-node cells. ○ = 800 r, bone marrow, and 17×10^6 lymph-node cells.

shortly after the irradiation. It may be postulated that the proliferation of the lymphosarcoma cells is stimulated in the irradiated host, possibly because of the depletion of the lymphatic tissues of normal lymphoid cells. Rapid growth of lymphosarcoma cells that survived the irradiation was suggested by one of our previous experiments, in which C57BL mice inoculated with relatively small amounts (5×10^4) of lymphosarcoma cells and treated with total-body irradiation and injection of isologous, homologous, or heterologous bone marrow died earlier of lymphosarcoma than the unirradiated, untreated controls.

Conceivably the injection of large numbers of normal isologous lymph-node cells might depress this proliferation stimulus. The lymphosarcoma cells, which survive the irradiation, would therefore multiply more slowly than in mice treated with bone marrow only. On the other hand, a predominant proliferation of normal lymphoid cells might inhibit the growth of the irradiated tumor cells by direct competition.

In a few animals of experiment 1 and in the majority of animals of experiment 2, treated with isologous lymphoid cells, the lymphosarcoma may be completely eliminated since the animals are still alive at the 100th day after the inoculation.

TABLE 1.—*Factors inhibiting growth of the lymphosarcoma as discussed in this paper**

	Damage to:	
	Host	Tumor
Irradiation	+	+
Antibodies	+	+
Wasting	+	Doubtful
Competitive proliferation	−	+

*A harmful effect to the host or the tumor is indicated by +.

Addendum

After the manuscript had been prepared, A. Lengerova and V. Zeleny reported, at the International Congress of Radiation Research, Burlington, Vermont, August 10 to 16, 1958,[5] secondary mortality in irradiated C57BL males protected with bone marrow of C57BL females. Since these findings could raise some objections against the interpretations of the experiments presented in this paper, the following comment seems relevant.

In our laboratory secondary mortality of irradiated C57BL males treated with bone marrow of C57BL females has never been observed. Experiments in which female C57BL lymph-node cells, with or without the addition of bone marrow, were injected into irradiated C57BL males have given no indication whatsoever of an immunological reaction of the female graft against the male host.

Although the rejection of (CBA ♂ × C57BL ♀)F_1 ♂ skin grafts by (CBA ♂ × C57BL ♀)F_1 ♀ hosts has occurred, secondary mortality after bone-marrow transplantation has not been found in this donor-host combination.

Since, moreover, the lymphosarcoma originated in a C57BL female, it seems unlikely that a sex-linked histocompatibility gene could have interfered with our results.

The Eichwald-Silmser effect has been investigated in our laboratory by Dr. O. Brocades Zaalberg. The results will be published in the near future.

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Cultivation of Human Breast Carcinomas [1,2]

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Summary

A method was developed for the cultivation of human breast carcinoma cells. This technique, taking advantage of the weak intercellular bonds within the tumor, harvests the cells "spilling" out of the tumor stroma when thin slices are cut with a razor blade. This suspension was sensitive to customary enzymatic concentrations which therefore cannot be used in the preliminary step of preparation. Agitation, even the slow motion of the roller drum, was unfavorable to the growth of the neoplastic cells which have to be explanted in resting tubes. Continuous cultivation of human breast carcinoma cells was achieved for the first time. The cultures, derived from a mammary duct-cell carcinoma of no special type, are now in their 6th month of life *in vitro* and extremely proliferative. A regular growth rate of the neoplastic cells appeared to be dependent on the presence in the medium of mucopolysaccharides introduced in the form of umbilical-cord extract. Human placental serum and beef amniotic fluid, whose high content in estrogenic hormones is well known, also had a beneficial effect. Folic acid in a 2.5 mM concentration appeared favorable to the growth of the cancer cells. However, a possible antagonism with cord extract was considered in relation to the spindle-shaped transformation which occurred in the epithelial cells when transferred from a folic acid medium to one enriched with cord extract.—**J. Nat. Cancer Inst. 21: 1131–1147, 1958.**

Although most of the human carcinomas have been successfully transplanted *in vitro*, the mammary carcinoma has remained a challenge for the investigator. The response of primary explants is highly unpredictable and depends mainly on the type of tumor.

The technical difficulties involved are apparent when the current literature is scanned. In the past 21 years, fewer than half a dozen reports can be found that refer to explantation and growth of human breast neoplastic epithelium. Cameron and Chambers (*1*) in 1937 were the first to give a detailed description of the behavior of these cells *in vitro*. They noticed the importance of the stroma structure which opposed epithelial migration, and succeeded in explanting primary ductal

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[2] This investigation was supported by research grant C-2520 from the National Cancer Institute, National Institutes of Health, Public Health Service, and was administered by Dr. M. R. Murray.

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carcinomas. The tumor cells spilled or migrated from the explants in the form of "hollow nodules" eventually spreading out into flattened sheets. In 1942, Coman (*2*) observed the weakness of the cells' contacts in breast carcinoma cultures. His observation was confirmed a few years later by Royle (*3*). More recently, Orr and McSwain (*4*), in a systematic attempt to grow human mammary carcinomas, obtained the highest percentage of success with ductal carcinomas. Even so, most of their cultures were mixed cultures in which the fibroblastic population was predominant.

Recent progress in research on the mouse mammary carcinomas makes it imperative to grow human breast tumors for comparative studies. Since viral induction of these neoplasms in the mouse has gained more support, the availability of human tissue-culture material for experimental purposes could be enlightening. Furthermore, valuable information might be obtained from its use in screening techniques for therapeutic agents. We are presenting in this report the development of a method for the isolation and growth *in vitro* of human mammary neoplastic epithelium, and our first results with its routine maintenance.

Methods of Cultivation

The tumor tissue was dissected from the breasts immediately after mastectomy. With the usual aseptic procedures, a 1 to 2 $cm.^3$ fragment, according to the volume of the tumor, was isolated and washed in Simms' saline solution (*5*). The fragment was kept overnight in this solution, which contained 1000 units of penicillin per ml., at 4° C.

The next day the fragment was transferred to a fresh saline solution and all the fat and connective tissue possible removed under the dissecting microscope. The clean fragment was washed rapidly in a new change of saline, and then placed in a Petri dish containing Simms' solution and 0.5 percent human placental serum.

With a sharp knife, thin slices were neatly cut from the main fragment, which was firmly anchored with forceps, and the slices themselves carefully chopped with the blade. When a cloud spread in the surrounding liquid, it indicated that a dense population of cells was liberated from the stroma. A microscopic examination of one drop of this suspension showed that it consisted almost exclusively of epithelial cells, some of them agglomerated in groups of 5 to 10; their morphologic appearance was recorded. The total cell suspension was then transferred to a centrifuge tube by means of a pipette.

Care was taken not to scrape the tissue fragment while preparing the cell suspension; a large number of cells could be injured and the tumor stroma loosened so that bundles of fibers are mixed with the suspension and are difficult to eliminate.

After a 3-minute centrifugation at 1000 rpm (International Centrifuge #240), most of the living cells were sedimented, and a large amount of debris was discarded with the supernatant. With a pipette, the pellet was carefully resuspended in a fresh volume of saline-placental serum,

centrifuged again under the same conditions, and finally resuspended in an adequate volume of nutritive medium.

A nutrient with a high hormonal content was specially designed for the maintenance and multiplication of these cells, as follows:

Eagle's medium (without glutamine) and beef amniotic fluid[3] mixed in equal parts represent the basic nutritive solution (BS). Human placental serum and umbilical-cord extract are diluted with it in these proportions:

For 100 ml. of medium,	
BS	55 ml.
Placental serum	25 ml.
Cord extract	10 ml.
Antibiotics	10 ml.

The cord extract prepared from human umbilical cords, according to a technique formerly described (*6*), brought the medium to a high level in mucopolysaccharides, which appeared to be essential for the survival of the cells. The antibiotics, penicillin and neomycin, were dissolved in Simms' saline solution in order to obtain a final concentration of 500 I.U. and 250 γ per ml. of medium, respectively.

Best results were obtained with resting tubes coated with a very thin film of plasma. Three drops of chicken plasma, used for mechanical purposes only, were distributed in each culture vessel and spread on the wall with a glass rod. The tubes were then kept on a 10° slant for 15 minutes, and the plasma that collected at the bottom was periodically aspirated with a pipette. When all the plasma was drained, 0.5 ml. of culture medium was introduced into each tube, rolled all over the wall, and pipetted out as completely as possible. The tubes were stoppered and placed on the roller drum at 37° C. for half an hour to insure a uniform coating. Coverslips, prepared in the manner described for cultivation of mouse mammary epithelium (*7*), proved useful for the fine cytologic study of the cell suspension in Maximow slides.

The prepared culture tubes were labeled, opened, and returned to the rack at a 5° slant. The neoplastic cells already in the culture medium were homogeneously resuspended with a graduated pipette and aliquots of 1 ml. were distributed in each tube. The containers were sealed and the resting rack kept *undisturbed* for 3 to 4 days at 37° C.

The medium was changed twice a week; 10 percent of the old medium was retained, but the basic nutritive solution (BS) of the new one was reduced 10 percent.

Transfers were made every 3 to 4 weeks. The cultures treated with a trypsin-Versene combination according to the procedure of Ian Leslie [4]

[3] Eagle's basal medium and beef amniotic fluid were obtained from Microbiological Associates, Bethesda, Md.

[4] Ian Leslie's procedure (personal communication to Dr. M. R. Murray):

a) The cultures are first washed 10 minutes in a calcium-magnesium-free saline solution, and then incubated 5 to 10 minutes in trypsin-Versene saline at 37° C.

A 100 ml. of trypsin-Versene saline is prepared as follows:

Bacto-trypsin (Difco Laboratories, Mich.) 2 percent in distilled water	2.5 ml.
Versene (Delta Chemical Works, N. Y.) 0.08 percent in distilled water	2.5 ml.
Calcium-magnesium free saline	95 ml.

b) After enzymatic action, the cell suspensions are pooled, spun gently in the centrifuge, resuspended in growth medium, and planted out.

gave homogeneous cell suspensions. These suspensions were easy to handle when making replicate preparations.

Cell counts were not made because many cells appeared to have reached the end of their life when first explanted—sometimes more than 50 percent did not survive. A count of the large cell population of the primary inoculum would therefore be misleading. However, accurate counts can be made from replicate cultures.

Results

When the original suspension was examined under the microscope, it could be seen that the cell population varied in the percentage of healthy cells. Some were globular, with a very refringent cytoplasm, and others were more granular and shapeless. The amount of healthy cells, which appears to be greatly dependent upon the general condition of the tumor, in turn influences the success of the culture. A systematic selection of tumors which yield the largest percentage of healthy cells can therefore be particularly rewarding. One breast tumor (BT 20), which was an advanced, infiltrating, duct-cell carcinoma of no special histologic type, gave a suspension composed of approximately 70 percent healthy cells and is presently in its 6th month of cultivation.

This neoplastic epithelium, apparently in pure form, is so far the only cellular line ever isolated from human breast tumors that can be maintained in continuous culture. The continuous cultivation of human breast cancer cells, which was the ultimate aim of this project, has been achieved through the progressive development of the technique described. Enzymatic digestion of tumor fragments before cultivation did not improve conventional techniques. Even with 5 medullary carcinomas, which are the most favorable type of tumor for explantation, only one outgrowth of fibroblasts was obtained (table 1). The first progress toward elimination of fibroblasts and of amorphous ground substance of the stroma was made in the next 3 attempts when scraping the tumor gave a cell suspension from which cultures were possible (table 2). From that moment pure epithelium could be obtained fairly regularly. Adjustment of the technique from scraping to cutting and use of resting tubes was made on our 15th attempt. Improvement of the medium by addition of amniotic fluid, folic acid, and cord extracts was developed in 11 attempts. The first replicate cultures were made successfully from BT 20 cells with cord extracts. Another successful maintenance and subcultivation of a particularly necrotic tumor (BT 23) was considered as evidence of the efficiency of the method.

Since common morphologic trends observed on 23 explanted human mammary carcinomas appear, at the cellular level, to be independent of the pathologic type of the tumor, we will give a more detailed description of our experiments with the BT 20 cells, which can be used as a point of reference for the others.

After distribution of the suspension in the culture tubes, the cells, either singly or in small groups, settle down rapidly on the plasma film,

TABLE 1.—*Attempts at dissociating stroma of human mammary tumors by enzymatic action*

Tissue from medullary carcinoma	Enzymatic treatment*		Culture type		Results and time of first outgrowth
	Collagenase† (*mg./ml.*)	Hyaluronidase‡ (*TRU/ml.*)	Roller tubes	Maximow slidess	
1	0.25 1-hour agitation	250 in medium	—	12	Fibroblasts, 5th day
2	0.25 ½-hour agitation	250 in medium	—	12	No growth
3	0.25 ⟷ 250 1½ hours' agitation		—	12	No growth
4	0.25 ⟷ 250 1½ hours' agitation	18 hours	6	12	No growth
5	Conventional cultures		—	12	Epithelium, 8th day

* The enzymatic solutions were made in Simms' saline. The cultures were fed in a medium composed of human placental serum diluted to 4 times its volume in Eagle's basal medium.

† Collagenase isolated from *Clostridium histolyticum* supplied by Dr. I. Mandl, Columbia University, N. Y.

‡ Testicular hyaluronidase (1000 TRU/mg.) supplied by Dr. K. Meyer, Columbia University, N. Y.

TABLE 2.—*Influence of medium on maintenance and growth of neoplastic cell suspensions obtained from human breast tumors*

Carcinoma	Attempts (*number*)	Suspension	Medium*	Culture type		Results and time of first outgrowth
				Roller tubes	Maximow slides	
No special type	1	Rolling fragments	Eagle—Pl.S, A.F.	12	—	Fibroblasts, 5th day
"	3	Scraping	Eagle—Pl.S., A.F.	30	64	Epithelium, 6th day
"	1	Cutting	Eagle—Pl.S., A.F.	Rest.† 12	32	Epithelium, 6th day
"	2	Cutting	Eagle—Pl.S., A.F., F.A.	Rest. 16	—	Epithelium, 6th–7th day
"	2	Cutting	Eagle—Pl.S., A.F., C.E.	Rest. 26	—	Epithelium, 2nd–7th day—*subcultures*
Fibrous	1	Cutting	Eagle—Pl.S., A.F., C.E.	Rest. 12	—	No growth
Necrotic	1	Cutting	Eagle—Pl.S., A.F., C.E.	Rest. 20	8	Epithelium, 3rd day—*subcultures*

*Medium constituents: Eagle's basal medium (without glutamine); Pl.S.=human placental serum; A.F.=beef amniotic fluid; F.A.=folic acid; C.E.=human cord extract.

†Rest.=Resting tube.

forming scattered colonies. Certain of the cells become attached first, while others remaining in suspension either die or eventually become part of the cellular colony. A variable amount of debris left from the processing of the tumor is gradually eliminated through successive washings and feedings. During the first days, the greatest part of the cell population is represented by the neoplastic epithelial cells, a few scattered fibroblasts, macrophages, and mast cells. The presence of red or white blood cells is seldom noted (fig. 1).

The epithelial cells, when healthy, appear as round, rather thick, elements delimited by a well-defined ectoplasmic membrane. Their cytoplasm is a clear, refringent, and relatively thin rim; the nucleus, quite thick and large, frequently contains more than one nucleolus. At this stage we are under the impression that in most of the neoplastic cells the volume of the nucleus is slightly superior to the volume of the cytoplasm.

Other cells, on the contrary, display a less well-defined cytoplasmic membrane, have a granular cytoplasm, and either a shrunken or vesicular nucleus. Most of these cells appear to be undergoing changes leading to disintegration. Some of them, however, can slowly recover and regain a healthy appearance.

In a period varying from 48 hours to 8 days, the neoplastic cells become flatter, while the nucleus maintains approximately the same features. The cytoplasm becomes clearer and extrudes protoplasmic extensions into the surrounding medium (fig. 2). This, in our experience, is the first sign of cellular activity.

After 8 days the cells multiply at a gradually increasing rate, forming epithelial sheets. These sheets are composed of one single layer of very thin cells presenting a generally polyhedral shape with significant variations in size. The nuclei, also variable in size, have well-defined nuclear membranes and prominent nucleoli. At this stage the nuclear/cytoplasmic ratio is reversed in favor of the cytoplasm. This transparent cytoplasm sometimes contains fine, brown granules. Frequently at the periphery of the epithelial sheets, the cells display large undulating membranes.

As multiplication of the cells progresses, an almost continuous epithelial sheet is formed by the merging of separate colonies (fig. 3). At this time the mitoses are so frequent that transfer to other tubes becomes imperative. In less than 48 hours the newly transferred cell suspension produces epithelial sheets with identical morphologic features and a high mitotic index (fig. 4).

These cells are very sensitive to the composition of the medium. For instance, when the cultures are fed with media that are not supplemented with human cord extract, the cells remain thick and globular with little tendency to spread and multiply (fig. 5). They occasionally form small epithelial sheets, but many show cytoplasmic vacuolization (fig. 6) and exhibit growth only for a limited period.

The cultures supplemented with cord extract always display a better growth. The epithelial sheets, monolayered and uniform, are composed

of cells that maintain constant morphologic features in spite of their volumetric differences (fig. 4). They appear to be able to grow indefinitely provided they are kept in a basic feeding solution of constant composition enriched with cord extract.

As folic acid is known to be essential for the growth of the female genital tissues, we attempted to promote a continuous cultivation of our cells by introducing massive amounts of this product in the medium. In such a series of experiments, cord extract was omitted to avoid the interference of its growth-activating properties. When folic acid was added to the medium in a 5 mM concentration, the cultures, at first enhanced, within 10 days showed a massive accumulation of crystals in their cytoplasm. Rapid deterioration and death followed. Better results are obtained with a 2.5 mM concentration. The growth rate is improved and the cells have a more healthy appearance (fig. 7). After 5 weeks, however, the epithelium reaches a static stage and, although the cells remain in a fairly good condition, no signs of multiplication can be detected.

When returned to a basic medium enriched with cord extract, the folic acid-treated cultures showed a most interesting morphologic modification. Immediately after removal of folic acid, the epithelial cells on the periphery of the colonies became more and more elongated, extruding slender processes joining identical structures from neighboring groups. Mitoses could again be seen, but the resulting cells assumed a spindle shape less and less distinguishable from fibroblasts. Four months after modification of the medium and 3 transfers, they appeared as prolific fibroblast-like cultures (fig. 8). This is even more interesting, since the BT 20 cells from which they derive retained all the characteristics of a pure epithelium.

A similar morphology appeared spontaneously with another explanted breast carcinoma that had not been exposed to folic acid. The epithelial cells began to spread and flatten down after 10 days, then presented elongated shapes thereafter. They still appeared in that form 2 months after their explantation (fig. 9).

By exposing the BT 20 cells of typical epithelial behavior to a medium supplemented with both human cord extract and folic acid in a 2.5 mM concentration, the growth is at first improved. After a week, however, degenerative changes, characterized by an intense granularity and vacuolization of the cytoplasm, occur (fig. 10). The degenerative changes progressively result in the death of the cultures. This could indicate a possible antagonism between folic acid and cord extract.

Discussion

A large number of cells is necessary as the primary inoculum; therefore small tumors, sometimes almost completely removed by the biopsy procedures, cannot be used for tissue cultures.

The size of the tumor is not the only limiting factor; its structure also has a great influence on the amount and the quality of the cells that can

be harvested (figs. 11 and 12). Infiltrating carcinomas with a hard, desmoplastic stroma usually yields a very small amount of cells. These cells, although of good quality, are sometimes not enough to originate a strong, healthy culture. Big tumors with an extremely large amount of epithelial cells may not necessarily offer a better prospect. Frozen sections of tissue from biopsies often show that the tissue is formed by cells already undergoing necrosis (fig. 12). In this case the fate of the culture depends entirely, as mentioned earlier, on the ratio between healthy and necrotic elements. Medullary and intraductal carcinomas, which usually give the largest amount of healthy cells, therefore have been the most successfully explanted.

Most of the authors who attempted to grow the breast tumors followed conventional procedures for explanting fragments of neoplastic tissue in a plasma coagulum. Besides the fact that epithelial cells grow better on top of a coagulum rather than in it, they are trapped in the more or less dense stroma of the tumor itself. From such explants the connective tissue develops abundantly, whereas the epithelium, partly necrotic from the beginning, has little chance to migrate out and multiply. Furthermore, in comparison with our massive cell suspensions, only an extremely small amount of epithelium can be explanted with a tumor fragment. Since the ratio between healthy and nonvital cells proved to be so important, the frequent failure of the conventional methods can easily be understood.

One of the main characteristics of the neoplastic mammary epithelium is the great fragility of the cells. They are particularly sensitive to shaking or any kind of motion; to date, we have not been able to grow them in roller tubes. The resting tubes give the most satisfactory results. The Maximow slides, however, made with an identical cell suspension, generally show a very poor growth. It is possible that the greater surface of the tube cultures and the greater atmospheric volume favor, in this case, more active respiratory exchanges.

Considering the tumor stroma as the main barrier to the free development of the epithelium, we first made several attempts to remove it enzymatically. Since collagen and mucopolysaccharides are major constituents of this stroma, digestion with collagenases and hyaluronidases, separately or in combination has been tried in a variety of times and concentrations (tables 1 and 3). We eventually succeeded in eliminating the stroma after 3 hours of a combined enzymatic digestion at 37° C., with strong agitation. Solutions containing 0.25 mg. of collagenase and 0.5 mg. of hyaluronidase (1000 TRU/mg.) per ml. could eliminate it but destroyed the epithelial cells as well.

This is in contrast to what is observed with the normal mammary epithelium, which in our experience can withstand such a prolonged action under still more violent physical stresses (table 3). The normal mammary tissues also give rise to epithelial sheets earlier than the neoplastic ones. This greater resistance is due, in our opinion, to the fact that the normal cells cling more strongly to each other and form self-

TABLE 3.—*Further attempts at dissociating the stroma of neoplastic and normal mammary tissues by enzymatic action*

Tissue	Enzymatic treatment*		Culture type		Results and time of first outgrowth
	Collagenase (*mg./ml.*)	Hyaluronidase (*TRU/ml.*)	Roller tubes	Maximow slides	
Carcinoma; no special type					
1	0.25 ⟷ 500 18 hours' agitation		—	—	Total digestion
2	0.25 ⟷ 500 1-hour agitation		—	12	Fibroblasts, 5th day
	3 hours' agitation†		—	12	No growth
	5 hours' agitation†		—	12	No growth
3	0.50 ⟷ 250 45 minutes agitation		—	12	No growth
4	0.50 ⟷ 250 1-hour agitation		6	12	Fibroblasts, 5th day
Normal breast tissue					
1	0.25 — ½-hour agitation		—	12	Epithelium, 5th day
2	0.25 ⟷ 250 48 hours' agitation		—	12	Epithelium, 4th day

*Enzymatic solutions and culture conditions are the same as in table 1. The greater resistance of normal breast tissues to enzymatic action is apparent.

†Stroma eliminated, but the resulting cell suspension was in very poor condition.

protecting groups, whereas the cancer cells with very weak intercellular bonds have a tendency to separate.

The nutritional needs of these cancer cells are also of great interest. The requirement for cord extract appears particularly significant because of the cord's high content of mucopolysaccharides. It is known that a large number of acid mucopolysaccharides appear in the stroma of human breast carcinomas as in other malignant tumors (*8–11*). Although their formation at this location is not yet understood, their presence might be correlated with the neoplastic process. We therefore attempted to introduce them into the medium, since this could be another example of the close cellular relationships we stressed in an earlier publication (*12*).

It also appears from all the attempts we have made, that human, neoplastic mammary cells need a high concentration of estrogenic hormones to survive. The survival time obtained with human placental serum could be significantly increased by the addition of amniotic fluid. From the recent estimations of Diczfalusy and Magnusson (*13*) on human placental serum and amniotic fluid, this would double the estriol content in the medium.

As to the antagonistic action of cord extract *versus* folic acid, no satisfactory explanation can be given at present. It seems, however, that irreversible changes can be obtained in the morphology of the cells through

the interplay of folic acid and cord extract (acid mucopolysaccharides). This is what happened to our BT 20 cells whose typical epithelial population could be changed into spindle-shaped elements. Such changes should be kept in mind in relation to the spindle-cell type of carcinomas occasionally observed by pathologists and to the spontaneous sarcomatous transformations of experimental carcinomas reported in the current literature (*14*, *15*).

Note Added in Proof

Since the submission of this paper, the fibroblast-like cells shown in figure 8 have again reverted to their original epithelial morphology. Such modifications therefore not irreversible are determined by variations in the environmental factors.

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PLATES

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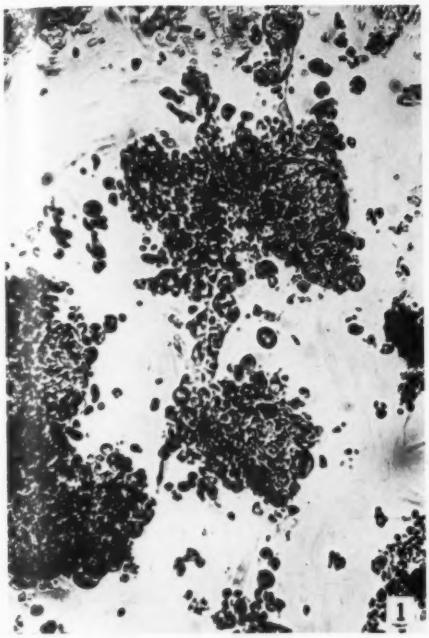
PLATE 114

FIGURE 1.—Agglomerated carcinoma cells, 2 days after explantation. A narrow strip of flattening cells can be observed in *center* of picture. The globular structure of the cells is evident. × 120

FIGURE 2.—Same culture 6 days after explantation. Typical epithelial sheets are developing, extruding protoplasmic extensions into the surrounding medium. × 120

FIGURE 3.—BT 20 cells, 15 days after explantation. Many of the epithelial colonies are merging. × 120

FIGURE 4.—BT 20 cells after 4 transfers and 4 months of cultivation *in vitro*. *Note* irregularity in size of cells. Some of them are multinucleated; others have a finely vacuolar cytoplasm. Mitoses are very frequent. × 120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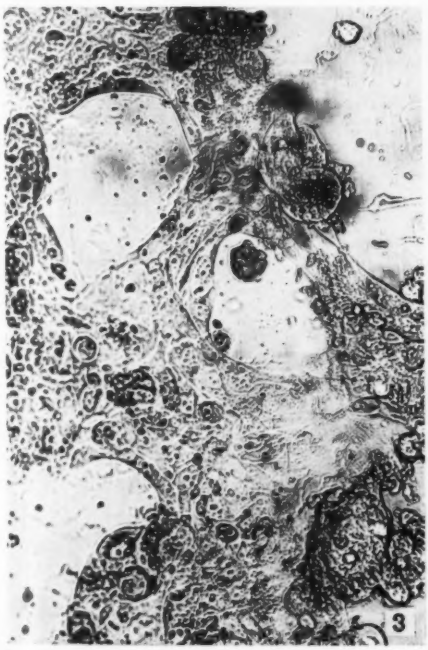
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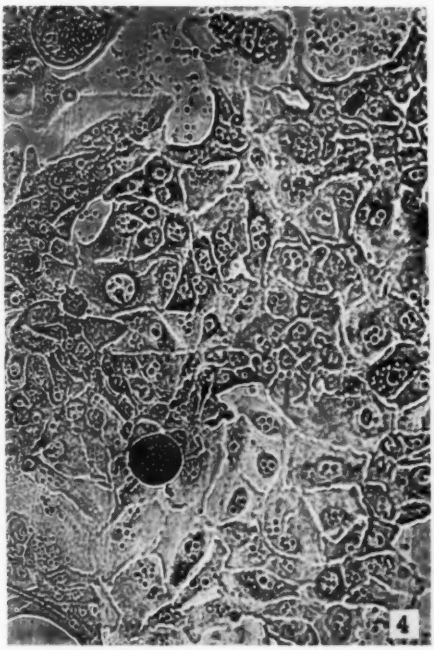
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PLATE 115

FIGURE 5.—Globular neoplastic cells fed in a medium deficient in cord extract. Particularly coarse nuclei are outstanding. Seven days' cultivation. × 375

FIGURE 6.—Groups of neoplastic cells with giant cytoplasmic vacuoles. The nucleus of each cell is compressed against the cell membrane where it can eventually be seen. Ten days' cultivation. × 630

FIGURE 7.—BT 20 cells maintained in medium with 2.5 mM of folic acid; no cord extract. Fifteen-day-old culture already in a static stage. × 120

FIGURE 8.—Fibroblast-like cells derived from cells shown in figure 7 when the cultures are returned to medium containing cord extract and no folic acid. This picture was taken 2 months after modification of the medium. × 120

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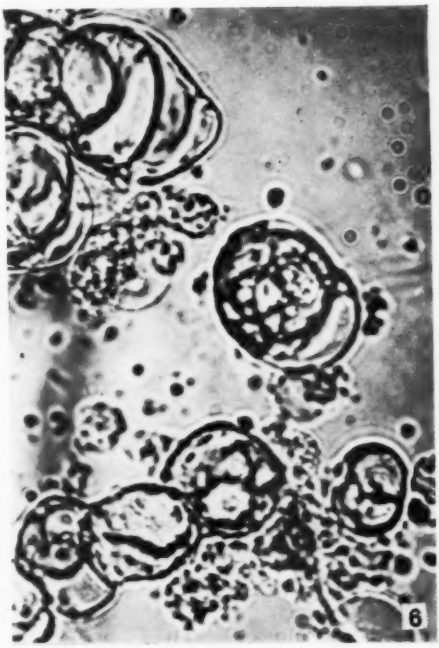
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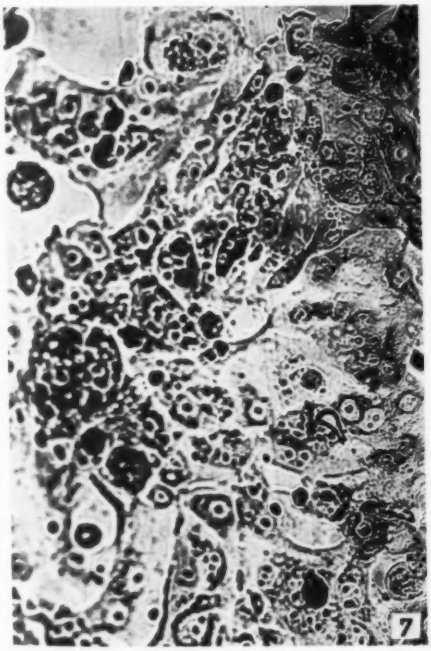
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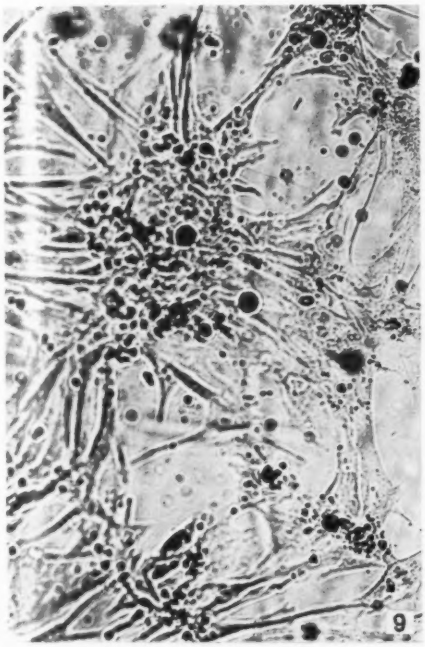
PLATE 116

FIGURE 9.—Fibroblast-like cells which appeared spontaneously after explantation of carcinoma cells from another tumor (BT 23). × 120

FIGURE 10.—BT 20 cells exposed to the combined action of folic acid (2.5 mM) and cord extract. Observe the increased vacuolization and granulation of the cytoplasm. Fifteen-day-old culture. × 120

FIGURE 11.—Histologic section of the BT 20 tumor. Very compact tissue made of turgescent epithelial cells with a dense nucleus. Hematoxylin-eosin. × 288

FIGURE 12.—Histologic section of the BT 23 tumor. Although very similar in its general appearance to the preceding one, most of the nuclei have their chromatin condensed on the nuclear membrane. This and the well-marked reticular structure of the cytoplasm are signs of an advanced necrotic stage. Hematoxylin-eosin. × 288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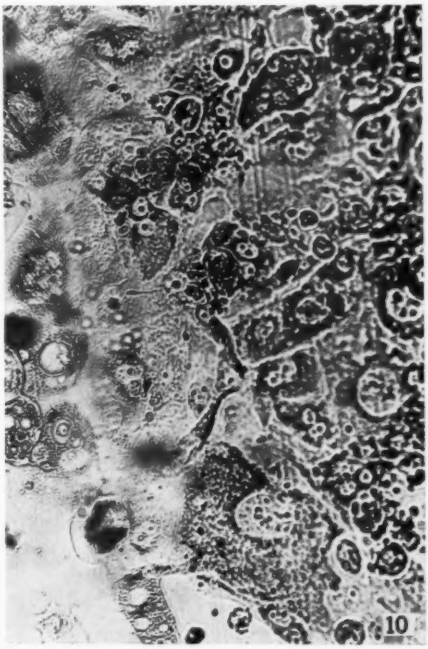
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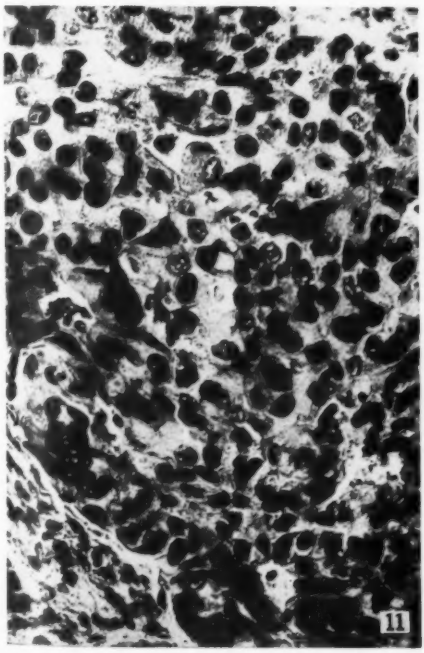
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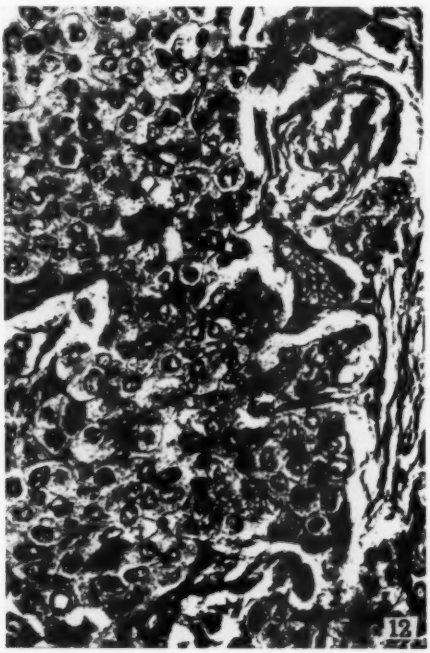
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Preservation of a Canine Thyroid Carcinoma in the Frozen State [1, 2]

M. W. Allam, L. S. Lombard,[3] E. L. Stubbs, *and* J. F. Shirer, *School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*

Summary

Canine thyroid carcinoma cells from a transplanted tumor, when preconditioned in 5.3 percent dextrose and preserved at −60° to −70° C. according to the method of Craigie, were found to retain a high percentage of viability. Tumor tissue thus preserved for 16 months was tested in pretreated puppies. Fifty percent of the injected animals developed tumors. No significant change was noted in the character of the tumor.—**J. Nat. Cancer Inst. 21: 1149–1150, 1958.**

Breedis and Furth (*1*) reported in 1938 on the feasibility of preserving a number of mouse tumors in the frozen state. Since then many investigators have published articles on the relative merits of the slow and the rapid freezing methods. The advantages of preservation at low temperatures as an alternative to serial transplantation of tumors are numerous. Craigie (*2*) stated that there is apparently a greater risk of changing the character of the tumor in serial transplantation for a number of years than in storing the tumor in a frozen state for a similar period. It is assumed that in preserving it no change takes place, but in many tumors this supposition has not been established.

In an attempt to determine the best method for preservation of a spontaneous canine thyroid carcinoma that was found to be transplantable in irradiated puppies (*3*), different modifications of the quick and the slow freezing methods have been tried.

Because of the relatively high percentage of viable cells (25%) retained by Craigie (*2*) when utilizing 5.3 percent dextrose solution to precondition tumor cells before submitting them to a slow freezing process, it was felt that his method offered definite advantages for the solid carcinoma that we were studying.

[1] Received for publication July 22, 1958.

[2] This investigation was supported by grant C-1720 from the National Cancer Institute, National Institutes of Health, Public Health Service.

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Materials and Methods

Tumor tissue was removed from 1 animal of the 36th transplant series on the 12th day of growth. The tissue was prepared for freezing according to the technique used by Craigie (*2*). The tumor was minced with curved scissors until the pieces would pass through a 10-gauge needle. The minced suspension was transferred by a 40-cc. syringe fitted with a 10-gauge needle to previously constricted 10-cc. pyrex test tubes and then sealed. After the tubes had remained at 4° C. for ½ to 1 hour, they were slanted for slow freezing in a carbon dioxide ice chest kept between —60° and —70° C.

Puppies to receive the frozen tumor tissue were selected and prepared as stated in a previous report (*4*). They were given total-body X-radiation doses of 350 to 400 r. The frozen tissue was thawed rapidly as described by Breedis (*1*), and each animal received a subcutaneous injection in the flank region of 2 to 4 cc., a dose which is 2 to 4 times that given when fresh material is used in serial passages.

Results and Discussion

The frozen tumor tissue was tested for transplantability at various intervals and was still viable after 16 months of preservation. Of the injected animals living 12 days or longer, 50 percent developed tumors, while 87 percent of animals injected with fresh tumor tissue in serial passages produced tumor growth. The microscopic appearance of the tumors closely resembled that of the serially transplanted tumors.

The tumor when frozen for 16 months did not show the initial rapid growth observed when unpreserved material was used. Distinct tumor nodules were first noted on the 25th to the 30th postinoculation day in animals receiving frozen material, whereas in the serial passages in which fresh tumor tissue was used, or even in the second series made from originally frozen tissue, distinct tumor nodules were observed on the 6th to the 7th day after transplantation.

Ten generations of tumor have been serially propagated with 16-month-old frozen material as the original source. The gross and microscopic morphology, take rates, and growth and regression characteristics in subsequent generations appeared to be essentially the same as noted in the serial passages started with fresh tumor tissue.

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Biologic Testing of 3,4,9,10-Dibenzpyrene [1,2,3]

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Summary

A reduction product of a vat dye, 3,4,9,10-dibenzpyrene, was tested for its carcinogenic activity. The potent activity of this compound, when administered subcutaneously, was shown by the high carcinogenic index and low latent period. The carcinogenic activity of 3,4,9,10-dibenzpyrene and two related polycyclic hydrocarbons was discussed in relation to the K-region hypothesis.—J. Nat. Cancer Inst. 21: 1151–1159, 1958.

In connection with a study of the structure of the quinonoid vat dye, Mayvat Brilliant Red AF,[5] Tilak, Venkataraman, and Unni (*1*) obtained a series of interesting polycyclic compounds, one of which was 3,4,9,10-dibenzpyrene—a hydrocarbon containing 6 fused benzene rings and 2 K regions. Biologic testing of this and other related compounds was undertaken as part of the study of the biologic activity of aromatic polycyclic compounds (carbocyclic and heterocyclic). The compounds were obtained from Dr. Venkataraman.

Materials and Methods

Mice used for the experiments belonged to the second generation of hybrids raised by crossing two inbred strains: XVII and C57BL, both resistant to spontaneous cancer of the breast. The hybrid stock was maintained on the optimum nutritive diet, described previously by Ranadive (*2*), and on water, *ad libitum*. The mice were housed in an air-conditioned room at 72° F. The only available group of mice consisted of 16 animals—8 males and 8 females—ranging in age from 4 to 5 months.

[1] Received for publication August 1, 1958.

[2] *Chemical Abstracts'* nomenclature: Dibenzo [a,l] pyrene.

[3] The investigations are being carried out under a program financed by the Council of Scientific and Industrial Research, New Delhi, India.

[4] We are greatly indebted to Drs. K. Venkataraman and B. D. Tilak for supplying the compounds under investigation, which were synthesized by them at the Department of Chemical Technology, University of Bombay, and the National Chemical Laboratory, Poona. We wish to express our grateful thanks to Dr. V. R. Khanolkar for his encouragement and valuable advice.

[5] A chloro-bromo derivative of 3,4,9,10-dibenzpyrene-5,8-quinone (text-fig. 1, I).

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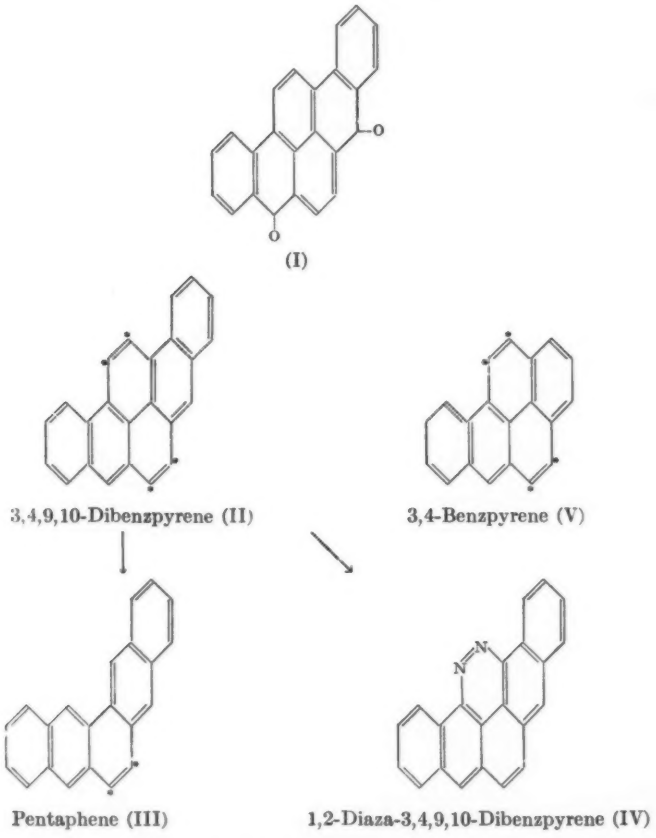

TEXT-FIGURE 1.—* = K region.

Since 3,4,9,10-dibenzpyrene (text-fig. 1, II) is not completely soluble in propylene glycol, a fine, ball-mill suspension was prepared. A single subcutaneous injection of 2 mg. of 3,4,9,10-dibenzpyrene suspension in 0.2 ml. of propylene glycol was administered per animal. The site of injection was inspected daily and the appearance of the palpable tumor was recorded. When the tumors attained appreciable size, the animals were killed and the tumor dissected out and fixed in Bouin's fluid. Six μ sections were stained by the routine method of hematoxylin and eosin, and Gomori's method for silver impregnation for the reticulum.

Results

Table 1 presents observations on individual experimental mice. All the 16 animals in the group receiving single subcutaneous injections of 2 mg. of 3,4,9,10-dibenzpyrene developed tumors at the site of injection. The tumors appeared within 2 or 3 months of treatment. The palpable tumors grew fast and attained a fairly large size within 2 to 3 weeks of their ap-

TABLE 1.—*Observations on experimental mice*

Animal No. (*sex*)	Age at treatment		Latent period palpable tumors* (*days*)	Age at death		Age of the tumor (*days*)	Fibrosarcomas	
	Month	Days		Month	Days		Spindle-cell sarcoma	Extreme pleomorphy
I 88♂	5	0	54	8	10	55	—	1
I 89♂	4	18	56	8	0	55	—	1
I 90♂	4	18	56	9	5	67	—	1
I 92♂	5	0	49	8	19	60	—	1
I 98♂	4	0	40	8	5	55	1	—
I 99♂	5	0	75	9	2	47	1	—
I 103♂	4	18	126	9	16	22	1	—
I 106♂	4	18	110	9	23	55	1	—
I 91♀	5	0	49	9	0	41	1	—
I 93♀	5	0	61	9	3	62	1	—
I 94♀	5	0	41	8	28	67	1	—
I 95♀	4	11	104	9	9	78	1	—
I 101♀	5	0	110	9	23	33	1	—
I 102♀	4	11	51	9	4	62	—	1
I 104♀	4	18	97	8	16	21	—	1
I 105♀	4	18	97	9	23	58	1	—

*Average latent period=74 days; Carcinogenic index=$\frac{\text{Tumor percentage}}{\text{Average latent period}} \times 100 = \frac{100 \times 100}{74} = 135.1$.

pearance. The average latent period was 74 days, and the carcinogenic index based on the percentage of tumor incidence and average latent period calculated according to the Iball method (*3*) turned out to be 135.1.

Histopathologic examination of the tumors revealed typical fibrosarcomas of the subcutis, varying in their histologic pattern and the grade of malignancy. Ten of the 16 tumors were classified as typical spindle-cell sarcomas, with closely packed spindle-shaped tumor cells. Solid masses of cells alternating with large and small whorls and interlacing bands of fibrous connective tissue were observed. Invariably the tumor cells exceeded the stroma and appeared slightly separated as though shrunken away from the intercellular stroma. The tumor cells ranged in size from large to medium, and in shape from blunt to long, narrow spindles. The cytoplasm was pale and eosinophilic and the nuclei were elongated and moderately hyperchromatic, with one or more large nucleoli. Mitosis was common and occasional giant cells were present (fig. 1). The remaining 6 tumors exhibited significant pleomorphy of cellular as well as nuclear outlines (fig. 2). The tumors were extremely cellular, the cells varying in shape from fusiform spindle to oval polygonal, with abundant eosinophilic cytoplasm and oval or spherical hyperchromatic nuclei. Some tumors had closely packed polyhedral cells, indicating a tendency toward sheet formation. There were many large mono- or multinucleate giant cells, and mitosis was frequent (fig. 3). These tumors were very active, with progressive dedifferentiation indicating a high grade of malignancy. Most of these tumors infiltrated into the muscle and into the hair follicles. Some were ulcerated and had leukocytic infiltration within 4 months of treatment. The reticulum was abundant—surrounding individual tumor cells (fig. 4).

Discussion

A preliminary set of hybrid mice that received a single injection of 3,4,9,10-dibenzpyrene developed 100 percent tumors within a short latent period of 74 days. The substance has thus proved to be a very potent carcinogen to the subcutis of mice. Experiments are now in progress to test the carcinogenicity of the substance on various other tissues.

The study of the carcinogenic activity of the compound has added interest in view of the fact that, earlier, Bonnet and Neukomm (*4*) had isolated a trace of this compound from neutral fractions of tobacco tar. The following year Lacassagne *et al.* (*5*) tested the carcinogenic activity of 3,4,9,10-dibenzpyrene and its derivatives. Our investigations confirm the results of the French workers in regard to the high carcinogenic activity of 3,4,9,10-dibenzpyrene.

Consideration of the chemical constitution of 3,4,9,10-dibenzpyrene and related products studied brings out some interesting points. Text-figure 1 gives the formulas of the parent quinone (I) and 3,4,9,10-dibenzpyrene (II); the former is prepared from Mayvat Brilliant Red by dehalogenation and the latter from (I) by reduction. Compound II has two bonds (K region) corresponding to 9,10 bonds of phenanthrene. These are contained in rings A and C and are represented by asterisks. The significance of an activated phenanthrene bridge (K region) in carcinogenesis by thiophene isosteres of carcinogenic hydrocarbons has been discussed earlier (*6–8*). It may be seen from text-figure 1 that 3,4-benzpyrene [6] (V) also contains two unsubstituted K regions which are also located as in 3,4,9,10-dibenzpyrene (II). Tilak *et al.* (*1*) obtained pentaphene [7] (III) and 1,2-diaza-3,4,9,10-dibenzpyrene [8] (IV) by a series of steps starting from 3,4,9,10-dibenzpyrene (II). Pentaphene is of interest as it is structurally related to 1,2-benzanthracene,[9] 3,4-benzpyrene, and 3,4,9,10-dibenzpyrene. It contains only one K region. Biologic testing of this compound (III) is under way.

1,2-Diaza-3,4,9,10-dibenzpyrene is an interesting derivative of 3,4,9,10-dibenzpyrene wherein one of the K regions (1,2 positions in ring A) has been replaced by a diaza group. This substance has been tested for the last 14 months, but so far no malignant tumors have developed. Ball-mill suspensions of the compound (text-fig. 1, IV) were administered in the same way as 3,4,9,10-dibenzpyrene in a group of hybrids. A typical tissue reaction resulting in formation of tiny nodules was observed at the site of administration. In most of the animals, however, the nodules either regressed or failed to show any progressive growth during the 12 months of observation. The animals are still living. A comparison of the activity of 3,4,9,10-dibenzpyrene indicates the significance of an unsubstituted ring, A, for the cancer-producing activity of these compounds.

[6] *Chemical Abstracts'* nomenclature: Benzo[*a*]pyrene.
[7] " " " Dibenzo[*b*,*h*]phenanthrene.
[8] " " " Dinaphto[1,2,3-*de*,3',2',1'-*ij*]phthalazine. *See* Ring Index (R. I.) #3602.
[9] " " " Benz[*a*]anthracene.

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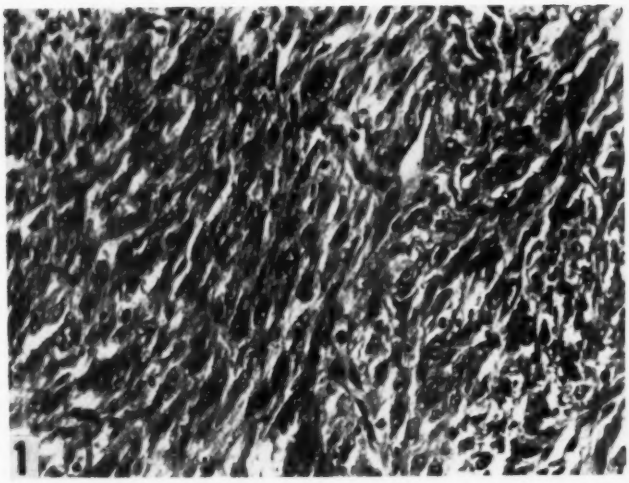
PLATE 117

FIGURE 1.—Histologic picture of a typical spindle-cell sarcoma induced by 3,4,9,10-dibenzpyrene in propylene glycol. × 290

FIGURE 2.—Histologic picture of a sarcoma exhibiting characteristic pleomorphy. × 290



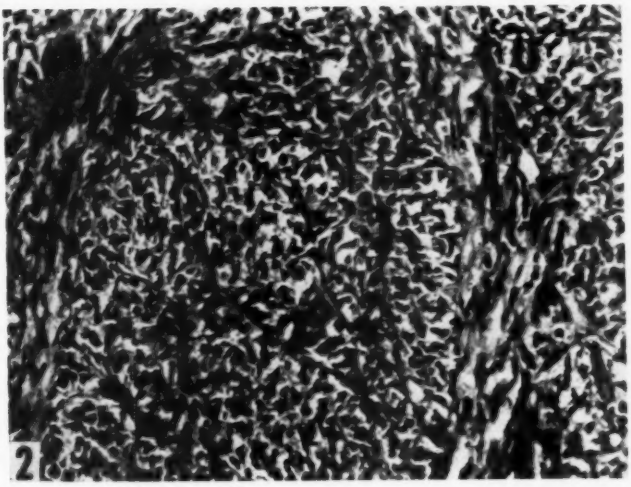


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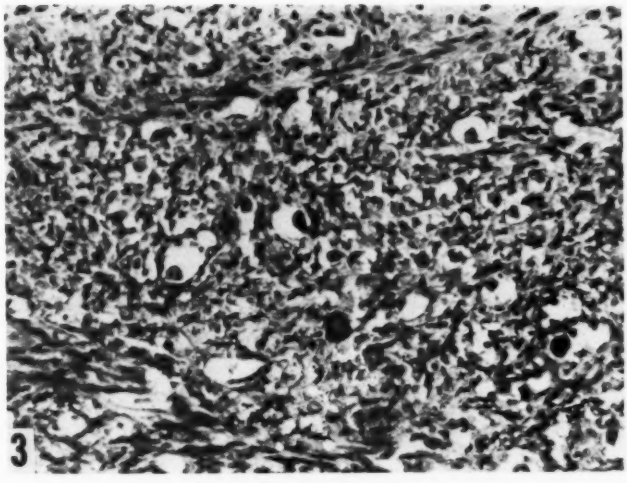
PLATE 118

FIGURE 3.—Section of a fast-growing pleomorphic sarcoma showing multinucleate giant cells. × 290

FIGURE 4.—Section of a sarcoma showing abundant reticulum—Gomori's silver impregnation technique. × 290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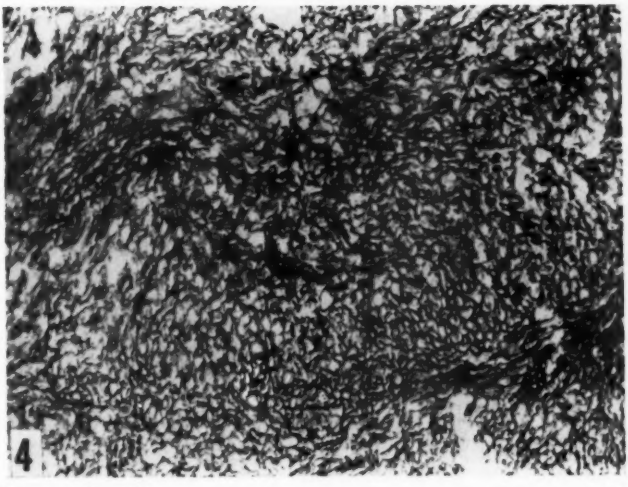
4

Role of the Pituitary in *N*-2-Fluorenyldiacetamide Carcinogenesis [1,2]

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Summary

It was demonstrated that hypophysectomy effectively prevented liver-tumor formation in male rats fed diets containing *N*-2-fluorenyldiacetamide (*N*-2-FdiAA). This prevention could not be reversed by administration of ACTH and insulin as occurs in azo-dye carcinogenesis. Hypophysectomy also *inhibited* ear-duct tumor formation in the *N*-2-FdiAA-treated animals. However, these rats developed a high incidence of tumors of harderian-gland origin. From evidence reported here, and from the results of other investigators, it is probable that fluorenamine carcinogenesis and azo-dye carcinogenesis may each be associated with different pituitary functions.—J. Nat. Cancer Inst. 21: 1161–1168, 1958.

The many tropic and growth regulatory functions of the pituitary would provide ample reason for the possible involvement of this gland in carcinogenesis. Indeed, in a large number of instances, endocrine imbalance, either in the pituitary or its several target organs, has been shown to initiate or to accelerate neoplastic formation within the pituitary or these target glands (*1*, *2*). Some investigators have also stated that hypophysectomy effectively inhibits the carcinogenic action of methylcholanthrene (*3*). However, the findings from several laboratories clearly indicate that cancer induction resulting from administration of the hydrocarbons is not prevented but only delayed (*4–6*).

In 1953, Griffin *et al.* (*7*) reported that hypophysectomy completely blocked liver-tumor formation in albino rats fed diets containing 3′methyl-4-dimethylaminoazobenzene[4] (3′MeDAB). This finding has been confirmed in several subsequent studies (*8–11*). In approximately 400 completely hypophysectomized rats, killed after 12 to 20 weeks of azo-dye administration, there was no gross, histologic, or chemical evidence of even the early precancerous liver alterations which are universally present

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[4] *Chemical Abstracts'* nomenclature: *N,N*-Dimethyl-*p*-(*m*-tolylazo)aniline

in intact rats after 3 weeks of azo-dye feeding. The livers of hypophysectomized rats maintained on the azo-dye regimen for more than 40 weeks were normal, although a low percentage of these animals has exhibited mild fibrosis and cirrhosis within restricted areas of the liver. It appears well established that this method of cancer induction is influenced to a considerable extent by pituitary function. Of the pituitary hormones, only adrenocorticotrophin and the growth hormone have been at all effective in restoring liver carcinogenesis in these hypophysectomized rats fed the azo dye (*11*). Recently, the combined administration of ACTH and insulin has produced more extensive restoration (*12*).

We wanted to ascertain whether or not hypophysectomy would inhibit liver carcinogenesis by other agents. *N*-2-fluorenyldiacetamide (*N*-2-FdiAA) was selected for this purpose since it has been established by Morris *et al.* (*13*) as one of the most effective of this group of liver carcinogens. In preliminary studies Hoffman (*14*) noted that removal of the pituitary prevented both the preneoplastic stages and liver-tumor formation when this compound was in the diet. Fluorenylacetamide and related compounds have also been shown to initiate tumor formation in the ear duct, mammary glands, lungs, and other tissues. We also wished to determine whether the pituitary would exert the same influence on carcinogenesis in these other tissues as it exerted on cancer development in the liver. Finally, studies were carried out wherein various pituitary and other hormones were administered to the hypophysectomized rats fed diets containing the *N*-2-FdiAA.

Methods

The methods followed were essentially the same as described in previous publications (*7-11*). Intact albino male rats were obtained from the Holtzman Rat Company, Madison, Wisconsin, and the hypophysectomized male albino animals were purchased either from the Endocrine Laboratories, Madison, Wisconsin, or the Hormone Assay Laboratories, Inc., Chicago, Illinois. Starting weights of the animals in these studies were 175 to 240 gm. for intact animals and 115 to 150 gm. for the hypophysectomized rats. All animals were maintained in wire-bottom cages, and semisynthetic diets the same as previously used (*15*) were fed *ad libitum*. The animals were in a room where the thermostat was kept at 26 to 28° C.

The *N*-2-fluorenyldiacetamide was prepared by acetylation of *N*-2-fluorenylacetamide, as described by Morris *et al.* (*13*), and added to the semisynthetic ration at a level of 0.05 or 0.04 percent. A total of 18 groups of either intact or hypophysectomized animals were maintained on this regimen for periods up to 30 weeks. In certain of the hypophysectomized groups either ACTH or a combination of ACTH and insulin was also injected, since these hormones, as demonstrated in previous studies (*8*, *10–12*), partially restored azo-dye carcinogenesis. When the animals were killed, gross, histopathologic,[5] and chemical studies were made on the livers and other susceptible tissues. Riboflavine determina-

[5] We are indebted to Dr. H. L. Richardson for this portion of the study. Further detailed reports will be published on this phase.

tions were carried out according to the procedure of Andrews (*16*) and Conner and Straub (*17*). Deoxyribonucleic acid was measured by the diphenylamine reaction as modified by Burton (*18*). Food intakes of the groups and individual animal weights were taken at monthly intervals.

Results and Discussion

During this study it was conclusively demonstrated that removal of the pituitary prevents liver-tumor formation, at least for 30 weeks, in male rats fed diets containing *N*-2-FdiAA. In addition, these hypophysectomized animals had a lower incidence of tumors of the ear duct and considerably increased numbers of tumors of harderian-gland origin. Intact rats fed this diet for periods of 6 to 22 weeks exhibited the progressive liver changes of fatty metamorphosis, cirrhosis, bile-duct proliferation, etc., as described by others (*13*, *19*, *20*), groups I to IV, table 1. A high incidence of liver and, also, ear-duct tumors was seen in the 18- and

TABLE 1.—*Effect of pituitary removal or restoration of pituitary hormones on cancer induction in rats fed diets containing* N-*2-fluorenyldiacetamide* (N-*2-FdiAA*)*

Group No.	Treatment	Duration of study	No. survived	Gross and histologic observations
		(*weeks*)		
I	Intact rats	6	6/8	Early nodular appearance of livers.
II	Intact rats	8	24/25	Livers—fatty metamorphosis, minimal cirrhosis.
III	Intact rats	12	9/12	Livers—moderate cirrhosis.
IV	Intact rats	18	15/18	Livers—bile-duct proliferation, stromal thickening, bile-duct cysts, hepatoma foci; 50 percent of survivors exhibited tumors of ear ducts.
V	Hypophysectomized rats on control diet	8	6/7	Livers normal.
VI	Hypophysectomized	8	6/10	Livers normal.
VII	Hypophysectomized + ACTH†	8	8/10	Livers normal.
VIII	Hypophysectomized	11 to 18	13/20	Livers normal.
IX	Hypophysectomized + ACTH†	11 to 18	14/20	Livers normal.
X	Hypophysectomized	26	5/10	Livers normal: 2 animals developed tumors of the harderian gland.
XI	Hypophysectomized + ACTH†	26	3/11	Livers normal.

*Purified diets containing 0.05 percent *N*-2-FdiAA were fed *ad libitum* to all groups except V.
†HP ACTHAR® Gel (Armour)—2 units administered subcutaneously every other day.

22-week groups (group IV, table 1 and group XII, table 2), which is in agreement with the observations of Morris *et al.* (*13*, *19*).

In contrast to the foregoing observations, we failed to initiate the usual precancerous liver lesions or to produce liver tumors in any of the *N*-2-FdiAA-fed hypophysectomized rats, even up to periods of 30 weeks (groups VI, VIII, X, table 1 and groups XIII, XIV, XVII, table 2).

TABLE 2.—*Further studies on the role of the pituitary on cancer induction in rats fed diets containing N-2-fluorenyldiacetamide (N-2-FdiAA)**

Group	Treatment	Duration of study (*weeks*)	Average initial weight/ average final weight (*gm.*)	No. survived	Gross observations and tumor incidence
XII	Intact rats	22	240/300	21/21	All livers were cirrhotic and enlarged: 21 animals with liver tumors; 4 ear-duct tumors.
XIII	Hypophysectomized	22	128/113	20/40	Livers normal. No ear-duct tumors. Seven eye (harderian-gland) tumors.
XIV	Hypophysectomized	22	115/104	6/15	Liver normal. Two eye tumors.
XV	Hypophysectomized + insulin†	22	115/106	3/5	Livers normal. No tumors seen.
XVI	Hypophysectomized + insulin† + ACTH‡	22	115/97	7/15	Livers normal. One eye tumor.
XVII	Hypophysectomized	30		0/30	Animals did not survive 30 weeks. Livers were normal at time of death. Three animals had developed eye tumors.
XVIII	Hypophysectomized + insulin† + ACTH‡	30		14/30	Livers normal. No ear-duct tumors. Six eye tumors.

*Purified diets containing 0.04 percent of *N*-2-FdiAA were fed *ad libitum* to all these groups.
†Insulin (Protamine, zinc—Lilly) 7.5 milliunits administered subcutaneously daily.
‡ACTH (Organon) 0.5 unit administered subcutaneously daily.

Livers of these animals retained the normal histologic appearance, and the riboflavine and deoxyribonucleic acid concentration did not show any significant change. Thus it would appear that some phase of pituitary function is essential at one or more stages of liver-cancer induction by this compound. Skoryna (*21*) has shown that removal of the pituitary in the later stages of *N*-2-fluorenylacetamide administration will still inhibit

the development of malignant liver tumors. In past studies it has been demonstrated that hypophysectomy effectively inhibits liver-cancer induction following administration of azo dyes (*8–11*). In this respect, liver-cancer induction by the azo-dye and fluorenamine compounds appears to be influenced by the same regulatory factors. Farber (*22*) has also noted similarities in the sequence of early histologic changes induced in the liver by the two types of compounds and by ethionine. As will be shown later, however, azo-dye and fluorenamine carcinogenesis may each be dependent upon different pituitary functions.

It is of interest that hypophysectomy resulted in a complete absence of ear-duct tumors in the rats fed *N*-2-FdiAA. This might be attributed to a longer latent period or to a direct inhibition of the carcinogenic sequence in these tissues. While these hypophysectomized animals exhibited no liver or ear-duct tumors, in contrast to the intact controls, there was a large increase in the incidence of eye tumors of harderian-gland origin (groups X, table 1 and groups XIII, XIV, table 2). Actually, we did not observe a single tumor of this type in the *N*-2-FdiAA-fed intact rats, while a total of 22 of these tumors were seen in the hypophysectomized rats. Morris *et al.* (*23*) reported a transplantable tumor that had its origin in the harderian gland when Buffalo-strain rats were fed diets containing *N*-2-fluorenylacetamide and, also, thiouracil, for 28 weeks. Subsequently, Engel and Copeland (*24*) described a high incidence of these tumors in A. E. S. strain female rats fed on low fat diets containing this same compound.

The influence of such factors as hormonal balance and diet became most apparent in the evaluation of these findings. Many of the dietary influences are considered in the excellent review on fluorenamine carcinogenesis by Weisburger and Weisburger (*20*). The food intake of the hypophysectomized rats fed the *N*-2-FdiAA diet was less than half that of similarly treated intact animals. This reduction in food intake may have had some influence upon the over-all carcinogenic mechanisms, inhibitory on some tissues and greatly accelerating in others, such as the harderian gland. Hormonal imbalances seem to be even more important. A combination of ACTH and insulin will restore liver carcinogenesis in hypophysectomized rats given 3′methyl-4-dimethylaminoazobenzene (*12*). The latent period of cancer induction is longer than in intact rats, but this combination of hormones is capable of producing a high tumor incidence in the hypophysectomized, dye-fed animals. Attempts to restore liver-cancer induction by administration of either ACTH or insulin, or both, in hypophysectomized, *N*-2-FdiAA-fed rats were unsuccessful (groups VII, IX, XI, table 1 and groups XV, XVI, XVIII, table 2). The livers maintained a normal histologic appearance and chemical composition even up to a total feeding period of 30 weeks. There were no significant differences in the incidence of harderian-gland tumors between the hypophysectomized or hypophysectomized-hormone treated animals.

From the preceding observations there is strong evidence for the importance of the pituitary in the chemical induction of tumors in the rat. The azo-dye process is partially restored by ACTH and insulin, indicating that carbohydrate metabolism may play an integral role. This is also supported by the observation of Salzberg and Griffin (*25*) that alloxan-induced diabetes also inhibited liver-tumor formation in azo-dye-fed rats. Spain and Griffin (*26*) and Chang *et al.* (*27*) have also reported histologic findings wherein the azo dyes affect glycogen deposition in the liver.

Liver-cancer induction by *N*-2-FdiAA appears to be regulated by other pituitary functions. There are numerous reports that thyroid activity may greatly influence liver-cancer induction by these agents. Paschkis *et al.* (*28*) reported that thiouracil inhibited liver-tumor formation in rats fed *N*-2-fluorenylacetamide. Later these same investigators (*29*) reported that uracil would abolish this anticarcinogenic action of thiouracil and that the effect should be attributed to interference with uracil metabolism rather than hypothyroidism. Bielschowsky and Hall (*30*) found that thyroidectomy completely inhibited liver-tumor formation in Wistar or Piebald strain rats fed diets containing *N*-2-fluorenylacetamide. In subsequent experiments, Bielschowsky (*31*) was able to induce liver tumors by painting 2-fluorenamine on the backs of thyroidectomized rats treated with growth hormone. Our present findings and those of other investigators clearly suggest that these pituitary factors which are implicated in liver carcinogenesis via the fluorenamine compounds are probably different from those which have been associated with azo-dye carcinogenesis.

Studies are in progress to determine whether or not administration of thyroxine or other hormones of the thyroid will partially restore the hormonal equilibrium in hypophysectomized rats fed *N*-2-FdiAA and thereby restore liver carcinogenesis. Bielschowsky (*20, 31, 32*) has, however, reported that while hypothryoidism may be an important factor in fluorenamine induction of liver cancer, the effects of the thyroid are mediated via the pituitary. The complex nature of these and other endocrine factors in the action of these agents is ably reviewed by Weisburger and Weisburger (*20*).

The observations obtained in this study only point to the complexity of carcinogenesis, and admittedly it may be difficult to extrapolate from hypophysectomized to intact animals. Nevertheless, these hypophysectomized animals have provided a valuable means for obtaining further insight into the multiple factors and the stepwise nature of the mechanism of cancer induction. Through this approach it has been shown that liver-cancer induction by azo dyes and by *N*-2-FdiAA are not identical and are influenced possibly at different stages by differing functions of the pituitary. The endocrine imbalances created by removal of the pituitary may have been responsible for the accelerated tumor formation in other tissues. From this approach we may reasonably expect to obtain information which may help in defining carcinogenesis in more specific terms.

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An Evaluation of the Eisenstaedt Serological Reaction in Cancer [1,2]

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Summary

The Eisenstaedt serological reaction as tested in this laboratory was found to be lacking in cancer selectivity and reproducibility.—J. Nat. Cancer Inst. 21: 1169–1171, 1958.

A flocculation test, based on the reaction of serum and an alcoholic extract of powdered spleens from patients who died of carcinomatosis, was described by Eisenstaedt and reported to be cancer-selective (*1*). He found 6.3 percent false positive results in testing 5,267 normal persons and 12.7 percent in 789 noncancerous patients, compared with 92.8 percent correct positive results in testing serums from 222 cancer patients. Because the alcoholic extract appeared to be unstable, a toluene extract of the splenic material was developed by Eisenstaedt and regarded by him as a stable antigen preparation. The present study is an evaluation of the reaction of this antigen in cancer diagnosis.

Method

In the flocculation test, 0.025 ml. of diluted antigen and 0.15 ml. of serum were mixed and agitated for 3 minutes on a Kahn shaker, and then 0.5 ml. saline was added. A flocculant precipitate in the mixture was a positive result. The tubes were examined within 1 or 2 minutes after addition of the saline. Although the published method (*1*) prescribed daily antigen titrations, Eisenstaedt's experience indicated that a 1 to 40 dilution of stock antigen could be used routinely and the titration omitted. Thus in our Series A the daily antigen titrations were omitted, and in Series B they were performed. In 33 of the 45 titrations in Series B (equivalent to 393 of 539 serums) the 1 to 40 dilution was found appropriate. Rapid pipetting and manipulation of the small volumes of

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[2] This investigation was supported by research grant CS-9051 from the National Cancer Institute of the National Institutes of Health, Public Health Service.

reactants specified in the method were found to be essential for reproducible results.

The tests were performed on serums from patients, in the University of Kansas Medical Center, whose diagnoses were not known at the time. Classification of each patient was made several months later after careful analysis of the completed hospital record. In Series A there were 51

TABLE 1.—*Incidence of positive results in groups of cancerous and noncancerous patients and in normal individuals*

Cancerous group	Series A*		Series B	
	Number positive	Total	Number positive	Total
Leukemia	2	7	5	11
Lung	2	6	2	4
Prostate	1	6	1	2
Bladder	1	4	0	0
Gastrointestinal tract	0	4	6	14
Uterus	1	3	2	6
Skin	1	4	1	6
Breast	0	3	1	3
Primary site undetermined	1	3	0	4
Miscellaneous	0	8	6	13
Cancer, treated, disappearance of tumor	0	2	0	6
Cancer, treated, without disappearance of tumor	1	1	0	2
Total	10 (20%) 51		24 (34%) 71	
Noncancerous group				
Heart disease	11	78	24	123
Minimal or no disease	9	83	10	98
Inflammation	14	75	25	72
Hyperplasia	2	15	5	22
Benign tumor	3	14	0	18
Pregnancy	1	12	1	5
Diabetes	0	5	0	16
Diseases of nervous system	4	18	2	17
Diseases of urinary tract	0	13	2	14
Diseases of gastrointestinal tract	6	21	8	31
Musculoskeletal diseases	1	11	4	14
Respiratory diseases	0	5	0	8
Miscellaneous	4	15	6	14
Total	55 (15%) 365		87 (19%) 452	
Total normal individuals	3 (15%) 20		2 (13%) 16	

*In Series A an antigen dilution of 1 to 40 was used throughout, whereas in Series B the appropriate antigen dilution was determined daily.

cancerous and 365 noncancerous patients, and in Series B, 71 cancerous and 452 noncancerous patients. There were 20 and 16 normal controls, respectively, in the two series. The types or sites of the tumor and the types of noncancerous disease are shown in table 1. The average age of the 64 cancerous men was 62 years and that of the 58 cancerous women, 58 years. In the noncancerous group, the average age of the 394 men was 53 years and that of the 423 women, 49 years.

Results

As shown in table 1, the incidence of false positive results in the normal and noncancerous groups was not greatly different, *i.e.*, 13 to 15 and 15 to 19 percent, respectively. There were 20 percent correct positive results in Series A (in which a 1 to 40 antigen dilution was routinely employed) and 34 percent in Series B (in which antigen was titrated daily). No particular type of cancerous or noncancerous disease had a significantly high incidence of positive results.

When the tests were performed without the antigen but with an equivalent volume of toluene, the incidence of positive results was slightly lower. Thus in Series A the incidence of positive results with the antigen in the normal, noncancerous, and cancerous groups was 15, 15, and 20 percent compared with 0, 10, 12 percent obtained without the antigen. In Series B the incidence of positive results with the antigen was 13, 19, and 34 percent compared with 0, 13, and 24 percent without the antigen.

During this evaluation, reproducibility was estimated by testing a serum 5 times in a single day. This was done on 7 different days with a total of 44 serums, which revealed 22 in which the 5 results agreed, 14 in which 4 of the 5 agreed, and 8 in which only 3 of the 5 results agreed.

In the preliminary evaluation with the powdered spleen as antigen, a positive result was obtained in 12 percent of 51 normal persons, 25 percent of 155 noncancerous patients, and 29 percent of 28 cancerous patients. These results were not appreciably different from those obtained with the toluene extract as antigen.

Discussion

No explanation can be offered for the wide difference between these results and those of Eisenstaedt. Possible sources of variation in the results include instability of the antigen and unrecognized variation between different lots of antigen. Factors relating to the technique of the test were discussed with Dr. Eisenstaedt and were not believed to account for these results. In our investigation neither the modified nor the original antigen gives useful results for cancer diagnosis. An improved antigen might give better reproducibility and improved cancer selectivity.

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